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**13. ABSTRACT** *(Maximum 200*

The long-range goal of this Application was to characterize the regulation of aromatase expression in the breast bearing a tumor. It included four specific aims: to quantify adipose tissue P450arom transcript levels in a large number of mastectomy specimens at various distances from the tumor; to determine alternative promoters that are used for aromatase expression in adipose samples proximal to a tumor; to characterize novel 5'-ends of P450arom transcripts in breast cancer tissues; to characterize tumor-derived factors that induce aromatase expression in the surrounding adipose tissue. We mapped distribution of adipose P450arom transcript levels in 18 mastectomy and 11 control reduction mammoplasty specimens. The highest levels were found in the adipose tissue proximal to a tumor. In the cancer-free breast, the highest transcript levels were localized to the upper and outer regions, which are the most likely sites of tumor development. Additionally, we demonstrated that the cAMP responsive promoters, II and I.3, are primarily used in the breast adipose bearing a tumor, whereas the cytokine/glucocorticoid-responsive promoter, I.4, is preferentially used in the cancer-free breast. In the breast cancer tissues, promoters II and I.3-specific transcripts comprise the majority of P450arom mRNA. The rest of the studies are detailed in the enclosed text.

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Serdar E. Bulun 10/09/96

PI - Signature Date

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# 5. INTRODUCTION

I prepared this Annual Report as a requirement of the Career Development Award entitled "Breast Cancer and Estrogen Biosynthesis in Adipose Tissue" funded by the US Army Medical Research and Material Command Breast Cancer Research Program. This report covers research for the period 9/12/95-9/12/96. The long term goal of this Application was to characterize the molecular and cellular mechanisms responsible for estrogen synthesis in adipose tissue surrounding a breast tumor. Since aromatase P450 (P450arom) catalyzes the conversion of $C_{19}$ steroids to estrogens, our studies focus on the expression of P450arom in adipose fibroblasts in relation to a tumor. During preliminary studies, we have detected the highest levels of adipose tissue P450arom transcripts in breast quadrants bearing a tumor compared with tumor-free quadrants.

The grant proposal included four specific aims: The **first aim** is to quantify adipose tissue P450arom transcript levels in an additional number of mastectomy specimens at various distances from the tumor using a novel quantitative RT-PCR method. We also proposed to quantify these transcripts in breast samples of women undergoing reduction mammoplasty to ascertain the distribution of aromatase expression. This aim also included determining the ratio of fibroblasts, the aromatase-expressing cell type, to mature adipocytes in these tissue samples. I indicated, in the previous year's annual report, that a large part of this specific aim had been accomplished. This specific aim has been fully accomplished now and will be detailed in **6. BODY**. The **second specific aim** included determination of the promoters that are used to express aromatase in adipose tissue samples proximal to a tumor. This involved, initially, development of a novel quantitative RT-PCR method to simultaneously determine untranslated 5'-ends of P450arom transcripts in adipose tissue samples using 4 different internal standard complementary (c) RNAs. This technology has been developed and published as indicated in the previous annual report. Recently, we applied this novel method to breast adipose tissue samples and found that certain aromatase promoters are preferentially used in the breast bearing a tumor. These promoters are used to a much lesser extent in the breast tissues of cancer-free women. The publication describing these results is in press. The results are detailed in **6. BODY**. **Specific Aim 3** involved characterization of novel 5'-ends of P450arom transcripts in breast cancer tissues and aromatase-expressing cancer cell lines, *e.g.*, MCF-7. The quantification of promoter-specific transcripts (see Specific Aim 2), however, indicated that the presence of a novel promoter in breast cancer tissues is highly unlikely, since the sum of promoter-specific transcripts in cancer tissues was roughly equal to total transcript levels that were determined independently. Therefore, the proposed studies in Specific Aim 3 were obviated. This is explained in **6. BODY** in detail. Finally, the **fourth specific aim** was to determine whether secretory factors of breast cancer cells induce aromatase expression in the surrounding adipose tissue and to characterize such factors. In this regard, we have characterized the expression of members of IL-6cytokine family in breast tumors. (We previously demonstrated that these cytokines markedly stimulate aromatase expression in adipose fibroblasts.) A description of our recently published paper is included in **6. BODY**.

## 6. BODY

**I.** As proposed in **Specific Aim 1**, we quantified P450arom transcript levels in 18 more mastectomy specimens in addition to the 15 that were previously reported in an attempt to duplicate our preliminary results. We used an alternative competitive RT-PCR assay that was recently developed in this laboratory. In a statistically significant number of cases, the highest P450arom transcript levels were found proximal to a tumor [Figure 4 in Reference (1)]. In this study, intratumoral P450arom transcript levels also were quantified and were not found to be statistically different from those in the proximal adipose tissue when normalized to total RNA. We also finished quantifying P450arom transcripts in breast adipose samples of cancer-free individuals as a control group (2). Therefore, the goal of the first specific aim was reached, and the association of tumor development or growth in relation to aromatase expression in proximal adipose tissue was established. In reference to **Specific Aim 1,** the following studies (1,2) have been published:

***In disease-free breasts, the highest fibroblast-to-adipocyte ratios and P450arom transcript levels were found in the outer regions*** (2). We determined distribution of P450arom mRNA levels and fibroblast to adipocyte ratios in disease-free breasts of premenopausal women between the ages of 19 and 42. Adipose tissue samples were collected from 3 regions of both breasts in 13 women undergoing reduction mammoplasty. Samples were taken from midportions of outer and inner regions, as well as from the midline above the nipple (designated as upper). Mode of sampling was dictated by the technique of surgery. Total RNA was isolated (n=11), and an hematoxylin and eosin-stained section was prepared (n=12) from the same sample from each region of both breasts. Overall, 67% of the highest fibroblast to adipocyte ratios and 64% of the highest P450arom transcript levels were detected in an outer breast region, whereas in only one patient, the highest values were detected in an inner region. Parametric analysis of variance (ANOVA) showed significant differences between the fibroblast content of the regions [p(F)=0.037]. This distribution pattern directly correlates with the most common or the least common sites of carcinoma in the breast in large series, which are the outer and inner regions, respectively. (In 11 of 15 patients of our previous study and 10 of 12 patients in O'Neill's study, the tumors were found in a lateral quadrant.) Moreover, a direct relationship was demonstrated between adipose fibroblasts and P450arom transcripts within the breast, in that regions with the highest fibroblast to adipocyte ratios contained the highest P450arom transcript levels (ANOVA of contrast variables, p=0.0009). This suggests that, similar to our previous findings in the breast bearing a tumor, adipose tissue aromatase expression in the disease-free breast is also determined by the local ratio of fibroblasts to adipocytes. We further conclude that since breast cancer occurs in regions of the breast with the highest levels of aromatase expression, the presence of high fibroblast content and P450arom transcript levels in the outer region of the disease-free breast may be of pathophysiological significance in the development of breast cancer.

**II.** To accomplish the studies proposed in **Specific Aim2**, we first developed a

novel competitive RT-PCR/SDS-PAGE method to quantify promoter-specific P450arom transcripts with unique 5'-ends (3), and this was described in the previous year's report. During the past year, we have completed the all of the goals in Specific Aim 2 by determining the distribution of promoter-specific transcripts in adipose samples from tumor-bearing breast specimens, as well as in tissues of cancer-free women as controls. The results will be published in November 1996 issue of Journal of Clinical Endocrinology and Metabolism (1). A summary is included below:

***P450arom promoters I.3 and II are primarily used in adipose tissue of the breast bearing a tumor, whereas promoter I.4 use is favored in the breast of cancer-free women (1).*** Three promoters (II, I.3, and I.4) are used for aromatase expression in human adipose tissue. First, we developed a novel RT-PCR assay in order to quantify promoter-specific P450arom transcripts using separate cRNA internal standards. This novel method was published separately (3). Then, in 18 mastectomy specimens, promoter use in adipose tissue samples was evaluated (1). Regardless of proximity to tumor, promoters I.3 and II (the cyclic AMP-inducable promoters) were found to be used in the breast bearing a tumor. This was observed in both adipose tissue samples, whether proximal or distal to a tumor, and in tumor tissues. On the other hand, in reduction mammoplasty samples from 9 cancer-free patients, promoter I.4 (the glucocorticoid plus cytokine-inducible promoter) was found to be primarily used for aromatase expression. We concluded that increased aromatase expression in breast adipose tissue bearing a carcinoma results from transcriptional transactivation of promoters I.3 and II. These two promoters are known to be used in adipose fibroblasts in response to treatment with a cAMP analogue. Therefore, it is conceivable that breast tumors secrete cytokines or growth factors that stimulate aromatase expression in stromal fibroblasts through a cAMP-dependent signalling pathway. Thus, **Specific Aim 2** was accomplished after completion of this proposed study.

**III.** ***Promoters I.3 and II are primarily used for aromatase expression in breast tumors (1)***. The goal of **Specific Aim 3** was to determine P450arom promoters in breast cancer tissues. In 9 breast tumor tissues, promoters I.3 and II were found to be primarily used for aromatase expression [Fig. 3 in Reference (1)]. Moreover, the quantities of the three promoter-specific transcripts (I.4, I.3 and II) with unique untranslated 5′-ends added up to total P450arom transcript levels (1,3). This indicated that initiation of transcription via these three promoters accounted for all of the P450arom transcripts found in breast cancer, and that the presence of a previously unidentified novel promoter would be highly unlikely. Therefore, a 5′-RACE analysis of P450arom transcripts in tumor tissue to search for a novel promoter was not necessary.

**IV.** The expression of IL-6 and related cytokines was investigated in breast tumors as potential secretory products, which are capable of stimulating aromatase expression in the surrounding adipose tissue. In the previous annual report, I described the studies that demonstrated that members of IL-6 cytokine family are potent stimulators of aromatase in adipose fibroblasts. Therefore, the following study further characterized the role of these cytokines in cancer epithelial-proximal stromal interactions:

***Expression of transcripts of IL-6 and related cytokines by human breast tumors, breast cancer cells, and adipose fibroblasts*** *(4).* The expression of transcripts

of cytokines of the IL-6 family has been examined in human breast tumors, breast cancer cell lines, and adipose fibroblasts, by means of RT-PCR amplification. Of the six breast tumor samples examined, all expressed transcripts encoding IL-6 and LIF. Four of the samples also expressed transcripts for OSM and IL-11, and three expressed the IL-6 receptor. Adipose fibroblasts expressed IL-6, IL-11 and LIF, but not the IL-6 receptor, consistent with previous conclusions that IL-6 activity in these cells required addition of IL-6 soluble receptor. In the case of T47D cells, expression of IL-11 protein was confirmed by immunotitration. Moreover, in these cells, expression of IL-11 transcripts was induced 3 fold by addition of $E_2$ to the culture medium. These results add credence to our previous proposal that breast cancer development is regulated in part by local autocrine and paracrine mechanisms via epithelial/mesenchymal interactions, in which estrogen produced by stromal cells surrounding the tumor acts to stimulate the production of growth factors and cytokines by the tumor cells. Some of these may act to stimulate further the growth and development of the tumor, while these or other factors may act on the surrounding mesenchymal cells in a paracrine fashion to stimulate aromatase expression. Thus a positive feedback loop is established which leads to the development and growth of the tumor.

As indicated in **5. INTRODUCTION**, the goals included in the broad category **Specific Aim 4** have been accomplished. We are, however, following our scientific instincts to continue our studies in the direction of further characterizing epithelial-stromal interactions in the breast bearing a tumor. Recently, prostaglandin $E_2$ was found markedly stimulate aromatase expression in adipose fibroblasts via promoter II. We will investigate prostanoid production in cancer tissues and prostaglandin E (EP) receptors in adipose fibroblasts.

We also completed the following study that was related to aromatase expression in the human adipose tissue. It bears this grant number and is summarized below. Cellular characterization of adipose tissue from various body sites of women (5). Adipose tissue is the primary site of estrogen biosynthesis in postmenopausal women. The two main histologic components of adipose tissue are mature adipocytes and fibroblasts. P450arom expressed in the fibroblast component of adipose tissue is responsible for catalyzing conversion of $C_{19}$ steroids to estrogens. We have previously demonstrated that, in women, aromatase expression in adipose tissue of various body sites increases with age and that aromatase expression in the buttocks is markedly higher than in the abdomen. In order to determine whether this age- and regional-dependent variation in aromatase expression is due to an alteration in the ratio of fibroblasts to mature adipocytes, we collected subcutaneous adipose tissue biopsy samples from 19 women (age range: 21-93) at the time of autopsy. Using a computerized image analysis system, we determined by morphometry the proportions of adipocytes, fibroblasts and vascular endothelial cells within histologic sections of adipose tissue from mid-abdomen, both breasts and both hips. The percentage of each cell component at each body site was expressed as the mean of triplicate replicates. Statistical analysis of our results did not indicate any correlation between advancing age and fibroblast to adipocyte ratios in the abdomen, hip or breast. Fibroblast to adipocyte ratios were found to be the highest in the breast and abdomen,

and significantly lower in the buttocks [p(F)=0.029]. No statistical differences were found between the breast and abdomen. These findings suggest that the increase in aromatase expression with advancing age and the higher aromatase expression in the buttocks compared with the abdomen in women may be due to alterations in specific signal transduction mechanisms rather than a simple increase in fibroblast numbers.

## 7. CONCLUSIONS

The studies summarized above all supported our working hypothesis. Regional differences in relative proportions of histologic components of the breast adipose tissue (*e.g.*, fibroblasts vs. mature adipocytes) may be the primary cause of estrogenic concentration gradients, since regions containing higher numbers of fibroblasts are the sites of increased aromatase expression. Although the initiating events are unknown, malignant cells in the regions displaying higher P450arom expression are more likely to proliferate. Secretory products of the tumor stimulated by estrogens may in turn further increase aromatase expression in the surrounding adipose tissue. These products will additionally stimulate proliferation of aromatase-expressing fibroblasts to generate a fibrous capsule around the tumor, *i.e.,* desmoplastic reaction. Estrogens will continue to positively influence neoplastic growth by increasing the expression of secretory products and their receptors in the tumor tissue. Thus a positive feed-back loop is established in which locally-produced estrogens and tumor-derived factors, *e.g.*, members of IL-6 cytokine family and/or prostaglandin $E_2$ act by paracrine and autocrine mechanisms to sustain the growth and development of the tumor.

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Publications by the Principal Investigator which are relevant to this research effort and which acknowledge this grant are listed:

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## 9. APPENDIX

Copies of the above publications are included in this annual report.

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# Use of Alternative Promoters to Express the Aromatase Cytochrome P450 (CYP19) Gene in Breast Adipose Tissues of Cancer-Free and Breast Cancer Patients*

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## ABSTRACT

Estrogen biosynthesis in adipose tissue has assumed great significance in terms of a number of estrogen-related diseases. Recent evidence suggests that estrogen synthesized locally in the breast is of singular significance in the development of breast cancer in elderly women. The biosynthesis of estrogen from $C_{19}$ steroids is catalyzed by a specific form of cytochrome P450, namely aromatase cytochrome P450 (P450arom; the product of the CYP19 gene). The human CYP19 gene comprises nine coding exons, II-X, and its transcripts are expressed in the ovary, placenta, testes, adipose tissue, and brain. Tissue-specific expression of the CYP19 gene is determined, at least in part, by the use of tissue-specific promoters, which give rise to transcripts with unique 5′-noncoding termini. Transcripts in adipose tissue contain 5′-termini derived from specific untranslated exons, corresponding to expression derived from the proximal promoter II and its splice variant I.3, as well as a distal promoter, I.4. The object of the present study was to determine the distribution of these various exon-specific transcripts in breast adipose tissues from cancer-free women undergoing reduction mammoplasty and from patients with breast cancer, because this would provide important clues as to the nature of the factors regulating aromatase expression in these sites. To achieve this, we employed competitive RT-PCR, utilizing an internal standard for each exon-specific transcript of the CYP19 gene, as well as for the coding region, to evaluate total CYP19 gene transcripts. In cancer patients (n = 18), total CYP19 gene transcript levels were significantly higher in adipose tissue proximal to a tumor in comparison with adipose tissue distal to a tumor, in agreement with previous findings. Moreover, total transcript levels were higher in breast adipose tissue of cancer patients in comparison with those of cancer-free individuals (n = 9), even when the adipose tissue from the cancer patient was taken from a quadrant with no detectable tumor. We observed that exon I.4-specific transcripts were predominant in breast adipose obtained from cancer-free women. In this tissue, promoter-II-specific and exon I.3-specific transcripts were present in low copy number. On the other hand, in breast cancer patients, CYP19 gene transcripts from breast adipose tissue had primarily promoter-II-specific and exon I.3-specific transcripts, whereas comparatively few transcripts had exon I.4-specific sequence at the 5′-terminus. We conclude that CYP19 gene transcription in breast adipose tissue of cancer-free individuals uses preferably promoter I.4, implicating a role of glucocorticoids and members of the IL-6 cytokine family in the regulation of this expression. On the other hand, the increased expression in breast adipose tissue bearing a carcinoma results from expression from promoters II and I.3, which are regulated by unknown factors acting via increased cAMP formation, which are presumably secreted by the tumor or associated cells. (*J Clin Endocrinol Metab* **81:** 0000–0000, 1996)

ADIPOSE tissue is the major site of estrogen biosynthesis in postmenopausal women (1, 2). In adipose tissue, estrogen biosynthesis increases with obesity but also with advancing age (3–8). Increased estrogen production in elderly obese women is believed to play a role in the pathogenesis of endometrial cancer (4). Furthermore, estrogen produced by adipose tissue within the breast may act locally to promote the growth of breast carcinomas (9). Estrogen biosynthesis is catalyzed by an enzyme known as aromatase cytochrome P450 (P450arom, the product of the CYP19 gene) (10–12). Aromatase is a member of the P450 superfamily of genes, which currently contains over 300 members in some 36 gene families (13). In the human, aromatase expression occurs in a number of human tissues and cell types, including syncytiotrophoblasts of the placenta (14), ovarian granulosa cells and corpus luteum (15, 16), testicular Leydig cells (17–19), various sites in the brain (20, 21), and in adipose tissue (2, 22–25). In women, aromatase activity and transcripts have been detected both in breast adipose and in breast tumor tissues. Within adipose tissue, aromatase expression occurs primarily, if not exclusively, in the fibroblasts (22, 26). A few years ago, we and others cloned and characterized the CYP19 gene, which encodes P450arom (11, 12). The coding region spans nine exons, beginning with exon II. Tissue-specific expression of the CYP19 gene is determined, at least in part, by the use of tissue-specific promoters, which give rise to transcripts with unique 5′-noncoding termini (27). Placental transcripts contain, at their 5′-end, untranslated exon I.1, which is located at least 40 kb upstream from the start of translation in exon II. This is because placental expression is

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driven by a powerful distal placental promoter, I.1, upstream of exon I.1. On the other hand, transcripts in the ovary contain sequence at their 5′-end that is immediately upstream of the start of translation. This is because expression of the gene in the ovary uses a proximal promoter, promoter II. By contrast, transcripts in adipose tissue contain yet another distal untranslated exon, I.4, located in the gene 20 kb downstream from exon I.1. A number of other untranslated exons have been characterized by us and others, including one that apparently is specific for brain (27–30).

A number of studies have attempted to relate tumor site to either aromatase activity (9, 31–34) or else aromatase expression (35) in adipose tissue of breast quadrants. In most cases (9, 31, 33, 35), a direct relationship has been found between the presence of a tumor and aromatase expression in the tumor-bearing quadrant. Moreover, we have demonstrated recently that, in women undergoing reduction mammoplasty, the highest adipose aromatase transcript levels were detected in lateral regions of the breast, the most common site where cancer develops (36). These results suggest that breast tumors may preferentially develop in areas with higher aromatase expression; and once developed, the tumors may secrete substances that influence aromatase expression in adjacent stroma. Recently, a number of cytokines have been found to stimulate aromatase expression in adipose fibroblasts (37) or else tumor-derived fibroblasts in culture (32). These include members of the class I cytokine family, namely interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), and leukemia-inhibitory factor (LIF). Several of these factors have been shown to be elaborated either by cells derived from adipose tissue (38) or else by tumor-derived fibroblasts (39) or breast tumor tissue (31, 38). The latter may include, in addition to transformed mammary epithelial cells, infiltrating macrophages and monocytes (31). Thus, it is possible to envision the establishment of a positive-feedback loop whereby fibroblasts surrounding a developing tumor produce estrogens that stimulate the tumor to produce a variety of cytokines and other factors. Some of these act to stimulate the further growth and development of the tumor in a paracrine and autocrine fashion. Additionally, these or other factors act to stimulate proliferation of the surrounding fibroblasts and expression of aromatase in these cells. Thus, a positive feedback loop is established via mesenchymal-epithelial interactions, which leads to the continuing growth and development of the tumor.

Sequencing of RACE-generated complementary DNA (cDNA) clones from adipose tissue reveals that, in addition to promoter I.4-specific transcripts, transcripts derived from promoter I.3 also are present (27). Interestingly, when adipose fibroblasts are placed in culture, the proportions of promoter I.4, II, and I.3-specific transcripts depend on the stimulatory factors present in the culture medium (27). Thus, when aromatase expression is stimulated by members of the class I cytokine family, such as IL-6 or IL-11, in the presence of glucocorticoids, the transcripts that are present are those derived from promoter I.4 (37). On the other hand, when expression is stimulated by dibutyryl cAMP in the presence or absence of phorbol esters, the transcripts that are present are those specific for promoters II and I.3 (27). Recently we have defined the second messenger pathway whereby class I cytokines stimulate aromatase expression via promoter I.4 (37). This stimulation is mediated by a Jak 1 kinase and a STAT 3 transcription factor that binds to a GAS (interferon-γ-activating sequence) element within promoter I.4 of the P450arom gene. On the other hand, factors that might regulate aromatase expression in adipose tissue via promoter II and I.3, presumably involving stimulation of adenylate cyclase, have yet to be defined.

As indicated above, expression of aromatase transcripts in breast adipose tissue seems to be higher in sites proximal to a tumor than at sites distal to a tumor (35). An important question then arises as to the nature of the 5′-termini of the aromatase transcripts that are expressed at higher levels at sites proximal to a tumor, as this might give clues to the nature of the factors produced by the tumor that are stimulating aromatase expression. To achieve this goal, it was necessary to develop a competitive RT-PCR method to amplify each of the exon-specific transcripts in the presence of the other transcripts, employing an internal standard specific for each exon-specific transcript. Validation of this methodology has been published elsewhere (40). In the present study, we report the quantitative detection of exon-specific aromatase transcripts in breast adipose and tumor samples derived from patients with breast cancer and from cancer-free individuals employing the above mentioned competitive RT-PCR method. This study has provided new insights into the regulation of aromatase expression in breast adipose at sites proximal and distal to a developing tumor.

## Materials and Methods

### *Tissue acquisition and processing*

Breast adipose tissue was obtained at the time of surgery from 18 women undergoing mastectomy for histologically confirmed breast cancer and from breast specimens belonging to 9 women undergoing reduction mammoplasty for symptomatic macromastia. Age, race, and tumor type of breast cancer patients are given in Table 1. Nine breast cancer patients had tumor in the upper lateral (outer) quadrant, 4 in the lower lateral (outer) quadrant, 2 in the upper medial (inner) quadrant. Three patients had a central tumor. This distribution of tumor location is consistent with published data of 5 different studies performed on a large number of patients in which it was found that tumors are located in the upper outer quadrant of 40–50% of breast cancer patients (41). Thus, our patient population was representative of the patient population in general. The ages of the women undergoing reduction mammoplasty ranged from 23–40 yr. Written consent was obtained before surgery, including a consent form and protocol approved by the Institutional Review Board for Human Research of The University of Texas Southwestern Medical Center. Whole breast adipose tissue and carcinoma samples were frozen in liquid nitrogen and stored at −70 C. Adipose tissue was sampled from each of the 4 quadrants in mastectomy specimens in the following fashion (Fig. 1): Sampling was performed 8–10 cm from the projection of the nipple at the posterior aspect of each specimen. In the quadrant bearing the tumor (Q1), fat was obtained 4–6 cm from the outer border of the neoplasm. Adipose tissue was dissected free of glandular and vascular tissue components of the breast. During reduction mammoplasty, because of the surgical technique, samples were obtained from 3 divided breast regions (Fig. 1): the superior region (S), the medial region (M), and the lateral region (L). Samples were taken from the center of each region.

### *RNA isolation and RT-PCR*

Human total RNA was isolated from the frozen tissues by the guanidinium thiocyanate-cesium chloride method (42). RT-PCR was performed according to a recently standardized competitive RT-PCR

**TABLE 1.** Clinical and pathological parameters of 18 breast cancer patients

| Patient | Age | Race | Tumor | Grade | Size (cm) | Axillary lymph node involvement |
|---|---|---|---|---|---|---|
| 1 | 64 | Black | IL | III | 3.5 | + |
| 2 | 41 | Black | IL | I | 2.5 | |
| 3 | 47 | Black | IL | III | 6.0 | + |
| 4 | 65 | Black | ID | | 3.2 | |
| 5 | 51 | Black | Medullary | I | 1.8 | |
| 6 | 51 | White | ID | II | 5 | + |
| 7 | 58 | Hispanic | ID | I | 1.3 | |
| 8 | 65 | Black | ID | II | 5 | + |
| 9 | 72 | Black | ID | II | 1.5 | |
| 10 | 55 | Asian | ID | I | 5 | |
| 11 | 53 | Black | ID | III | 4 | + |
| 12 | 39 | Black | ID | III | 2.3 | + |
| 13 | 54 | Black | ID | III | 3.5 | + |
| 14 | 59 | Black | ID | III | 1.8 | + |
| 15 | 36 | Hispanic | ID | I | 1 | |
| 16 | 84 | Black | IL | III | 2.8 | + |
| 17 | 47 | Hispanic | ID | III | 4 | + |
| 18 | 50 | Black | ID | II | 3 | + |

IL, Infiltrating lobular; ID, Infiltrating ductal

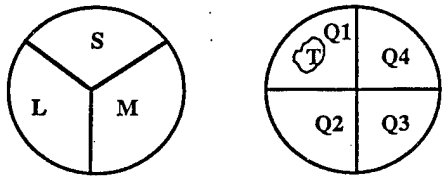


FIG. 1. Location of breast adipose tissues obtained from cancer-free subjects (A) and breast cancer patients (B). L, lateral; S, superior; M, medial; T, tumor; Q1, tumor bearing quadrant; Q2, Q3, Q4, nontumor-bearing quadrants.

method that we developed for this purpose (40). RNA was treated initially with DNase I to remove any contaminating DNA. Total RNA was then reverse-transcribed, using random hexamers. Complementary DNA was used in subsequent PCR amplifications for 25 cycles. Specific 5′-end primers were used to amplify the various 5′-termini. The 3′-end primer was the same in all reactions (40). A trace amount of [$^{32}$P] dCTP was added to each PCR mix. The reaction products were analyzed on 4% nondenaturing polyacrylamide gels. Gels were either autoradiographed with x-ray film or scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantitatively analyzed using ImageQuant software. For quantitative analysis, specific internal standards generated by inserting a 27-bp random AT-rich region (the looped-oligo method) (40) were employed for exon-specific transcripts containing each 5′-terminus, as well as for the coding region. The latter represents all of the P450arom transcripts present in the sample, regardless of the 5′-termini. The expected sizes of amplification products are given in Table 2. Quantification of each transcript in the cancer-free breast tissue was performed using two or three different copy numbers of each internal standard, together with the fixed quantity of target RNA. The copy number of internal standard that yielded an intensity value equivalent to that of the target was considered to be equivalent to the copy number of target transcripts corrected for size difference; for details, see Ref. 40. In mastectomy specimens, specific transcript levels were expressed as arbitrary units normalized to GAPDH. To check the integrity and comparative quantity of RNA used in amplification of CYP19 gene transcripts from all samples, transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, were amplified by the RT-PCR method described earlier (40).

### *Statistical analysis*

We used three statistical methods in the following order to analyze the data: Initially, two-way parametric repeated measures ANOVA was applied to determine overall significance. A Newman-Keuls multiple-comparisons test was then applied to evaluate levels of significance among specific transcripts (I.3, PII, I.4) within each group of women (cancer-free *vs.* women with breast cancer). To evaluate the interaction between group and specific transcript distribution, a two-way parametric repeated-measures ANOVA model was applied. Finally, within-subject paired *t* test with Bonferroni adjustment was used to evaluate interaction of specific transcripts in quadrants (quadrant bearing a tumor *vs.* average of nontumor-bearing quadrants).

**TABLE 2.** Expected sizes of PCR-amplified products

| Alternative Exon I (5′-Termini) | Target | Internal standard |
|---|---|---|
| Exon I.4-Exon III | 294 bp | 321 bp |
| Exon I.3-Exon III | 289 bp | 422 bp |
| Exon II-Exon III (promoter II-specific) | 305 bp | 332 bp |
| Exon II-Exon III (coding region) | 194 bp | 221 bp |

## Results

### *Alternative transcripts of the CYP19 gene in cancer-free subjects*

Firstly, we examined the distribution of P450arom (CYP19 gene) transcripts with unique 5′-ends in breast tissue of a group of cancer-free women (n = 9) who underwent a reduction mammoplasty procedure. The adipose tissue was sampled from roughly equal regions of both breasts, namely from superior (S), lateral (L), and medial (M) regions (Fig. 1). To quantify the transcripts, known copy numbers of each specific internal standard transcript were added with the target RNA (40) in the RT-PCR reaction. Fig. 2 depicts a representative experiment employing one sample and shows the amplification of the coding region (total transcripts) and transcripts specific for three 5′-termini: promoter II-specific, exon I.3-, and exon I.4-specific, each with their respective internal standards. By adding the copy numbers of all three exon-specific transcripts, good agreement was obtained with the copy number of the coding region (Table 3), indicating that these three species accounted essentially for all of the P450arom transcripts present in the RNA. We also performed experiments without internal standards, although values were still normalized to GAPDH. Then we compared both

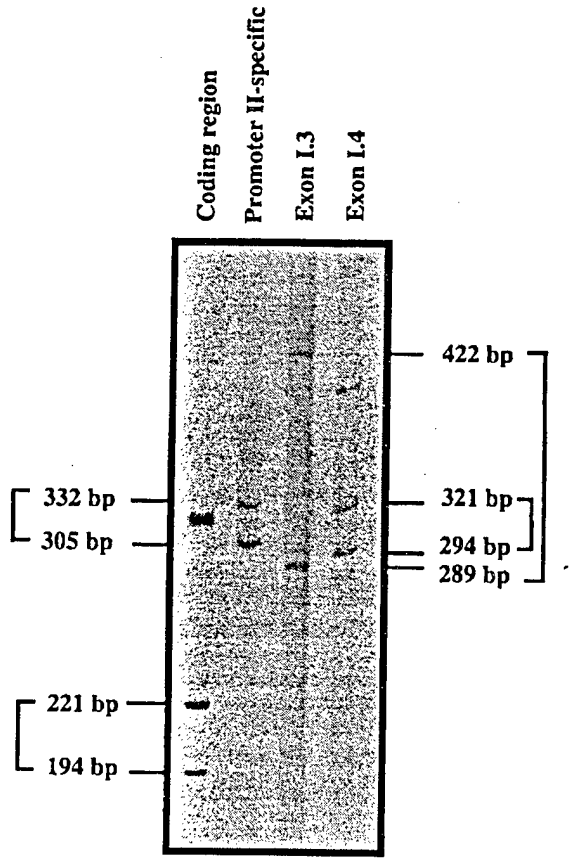


FIG. 2. Amplification of coding region (total CYP19 gene transcripts) and exon-specific CYP19 gene transcripts from RNA isolated from adipose tissue of cancer-free subjects, together with the corresponding internal standard. cDNA from 1 $\mu$g RNA was used for each reaction with respective internal standards. The experiment shown is representative of experiments performed using RNA from adipose tissue of cancer-free subjects. Each target and its corresponding internal standard are linked for illustrative purposes by brackets. The size difference between target amplified products and internal standard was 27 bp (Table 2) except for exon I.3. For exon I.3, internal standards were synthesized from full length exon I.3 sequence, whereas amplified product from target RNA was truncated exon I.3. For details, see references (27, 40).

sets of data (employing internal standard and without internal standard). We found a statistically indistinguishable ratio of each exon-specific transcript to total transcripts whether internal standards were employed or not. Thus, from the data obtained from cancer-free women, we were able to demonstrate that in our hands (although the use of the internal standards was necessary for absolute quantification purposes), for the purpose of comparing levels of transcripts to one another, it was not necessary to employ these standards. Exon-specific transcript levels measured in the absence of internal standards were expressed as percent of coding region transcripts (total transcripts), and this is shown in the *left-hand panel* (CF, *i.e.* cancer-free) of Fig. 3. A two-way parametric repeated-measures ANOVA revealed significant differences among the levels of these three types of P450arom transcripts [$P(F)<0.0001$]. A Newman-Keuls multiple-comparisons test showed that, overall, I.4-specific transcript levels (mean = 3.46) were significantly higher ($P < 0.0001$) than both 1.3- (mean = 2.11) and PII-specific (mean = 1.47) transcript levels. No statistically significant difference was detected between I.3- and PII-specific transcript levels (*first set of columns*, Fig. 3). For illustrative purposes (Fig. 3), exon I.3-, PII-, and I.4-specific transcripts are expressed as percentages of the total transcript levels instead of as arbitrary units. (The statistical significance levels were enhanced over parametric analysis after this conversion.) A trend was observed in both breasts, indicating higher total transcript levels in superior and lateral regions, compared with the medial region. This observation is in agreement with a previous study (36).

*Total CYP19 gene transcript levels in patients with breast cancer*

The fact that it was not necessary to employ internal standards for the purpose of comparing levels of transcripts to one another was a critical point in analyzing the samples obtained from cancer patients, because insufficient tissue was available from these individuals to allow the use of the internal standards in the methodology (although the GAPDH normalization was still employed). Thus, we were unable to determine the absolute copy number of CYP19 gene transcripts. Instead, we determined the comparative values of transcript levels in the samples obtained from breast cancer patients. From the 18 breast cancer patients, breast adipose tissue was sampled from four roughly-equal regions (quadrants Q1, Q2, Q3, Q4, Fig. 1). The tumor-bearing quadrant was designated as Q1, whereas Q2-Q4 were tumor-free. Tumor RNA [neoplastic tissue (T)] was also processed. Thus, five samples were obtained from each patient. In nonneoplastic adipose tissue proximal to the tumor (tumor bearing quadrant, Q1), total (coding region) CYP19 transcript levels were significantly higher ($P = 0.018$) in comparison with levels found in tissue distal to the tumor (average of three nontumor bearing quadrants, Q2-Q4) (Fig. 4). The differences between the tumor (T) *vs.* Q1, and tumor (T) *vs.* Q2-Q4, were not significant (Fig. 4). A within-subject paired *t* test was used for this statistical analysis. Total P450arom transcript levels in quadrants Q2-Q4 were found to be significantly higher ($P = 0.03$; after Bonferroni adjustment) from those in adipose tissue from cancer-free subjects (CF) (Fig. 4).

*Alternative transcripts of the CYP19 gene in breast cancer patients*

The distribution of exon-specific transcript levels in patients with cancer was quite different from that observed in cancer-free individuals (Fig. 3). Transcripts containing exon I.3-specific and promoter-II-specific sequences were the most abundant (Fig. 3), whereas exon I.4-specific transcripts were present but in lower copy number (Fig. 3). Overall, a two-way parametric repeated-measures ANOVA revealed significant differences [$P(F) = 0.0004$] among these three groups of transcripts. Subsequently, Newman-Keuls multiple-comparisons analysis showed significant differences between I.3- (mean = 11.41) and I.4- (mean = 3.85) specific transcripts ($P = 0.002$), as well as between PII- (mean = 7.97) and I.4-specific transcripts ($P = 0.008$) but not between I.3- and PII-specific transcripts. The within-subject paired *t* test (with

TABLE 3. Number of transcripts present in 1 μg of RNA isolated from breast adipose tissue of cancer-free subjects

| Variable[a] | Coding region | Promoter II-specific | Exon I.3-specific | Exon I.4-specific |
|---|---|---|---|---|
| RS | 4.15 ± 1.91 (9) | 0.93 ± 0.55 (8) | 1.08 ± 0.55 (8) | 2.11 ± 0.89 (8) |
| RM | 3.15 ± 1.67 (8) | 0.89 ± 0.44 (8) | 0.92 ± 0.59 (8) | 1.45 ± 0.72 (8) |
| RL | 3.32 ± 1.78 (9) | 0.57 ± 0.38 (9) | 1.01 ± 0.89 (9) | 1.92 ± 1.07 (9) |
| LS | 3.41 ± 1.83 (9) | 0.71 ± 0.48 (9) | 0.96 ± 0.55 (9) | 1.78 ± 0.97 (9) |
| LM | 3.35 ± 1.29 (9) | 0.74 ± 0.46 (8) | 1.03 ± 0.34 (9) | 1.44 ± 0.38 (9) |
| LL | 3.66 ± 1.29 (9) | 0.66 ± 0.24 (9) | 1.34 ± 0.72 (9) | 1.70 ± 0.56 (9) |

Data are presented as mean ± SEM (n) × $10^5$, where n = no. of subjects.
[a] R, right; L, left; S, superior; M, medial; L, lateral.

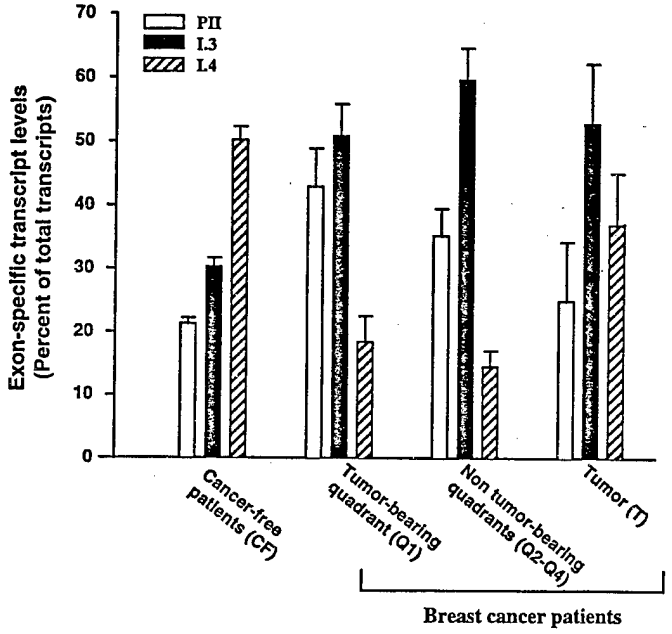


FIG. 3. Amplification of exon-specific CYP19 gene transcripts in cDNA from 1 μg RNA isolated from different breast quadrants of cancer patients and cancer-free individuals. Data are presented as mean ± SEM of percent of total transcripts (*i.e.* coding region) and are normalized to GAPDH. PII, promoter II-specific transcripts; I.3, exon I.3-specific transcripts; I.4, exon I.4-specific transcripts. Q1 and Q2-Q4 are the different quadrants of cancer patients. T, Tumor (n = 9); Q1, tumor-bearing quadrant (n = 15); Q2-Q4, average of nontumor-bearing quadrants Q2, Q3, and Q4 (n = 15). CF, Average of 3 regions from both breasts of 9 cancer-free patients. Percentage was calculated considering coding region as 100.

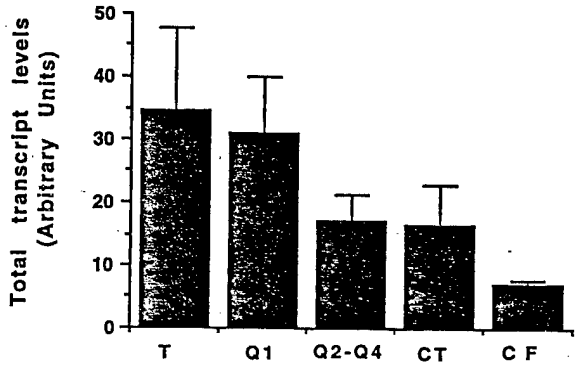


FIG. 4. Amplification of coding region (total transcripts) of CYP19 gene transcripts in cDNA from 1 μg RNA isolated from different breast quadrants of cancer patients and cancer-free individuals. Data are presented as mean ± SEM and are normalized to GAPDH. CT, average of four quadrants of 3 cancer patients with a centrally-located tumor. Other abbreviations are as in the legend to Fig. 3.

Bonferroni adjustment) also was applied to evaluate differences among transcripts containing I.3 and I.4, I.3 and PII, and I.4 and PII (expressed as percentages of total transcript levels, Fig. 3) in each quadrant [Q1, average of Q2-Q4 and tumor (T)]. It was found that I.3-specific transcripts were significantly higher than those containing I.4 in Q1 ($P$ = 0.001) and in Q2-Q4 ($P$ = 0.004) and not significantly higher in tumor (T) (Fig. 5). I.3-specific transcripts were also significantly higher than those containing PII in Q2-Q4 ($P$ = 0.004). Transcripts containing I.4 were significantly lower than PII-specific transcripts in Q1 ($P$ = 0.024) and in Q2-Q4 ($P$ = 0.054) (Fig. 5). There were no significant differences among tumor (T) and Q1, tumor (T) and Q2-Q4, or Q1 and Q2-Q4 for any of the three exon-specific transcripts.

A two-way parametric repeated-measures ANOVA model was applied to evaluate the effect of group (cancer-free women *vs.* women with breast cancer) on the distribution of exon-specific transcripts (I.3, PII, and I.4) in the breast. A significant interaction was found between the group of women and the transcript type ($P$ = 0.0009). This statistical significance was amplified when arbitrary units for transcript levels were converted to express each specific transcript as a percent of the total transcript levels.

Three cancer patients had tumors located in the center portion of the breast (CT; Fig. 4). No significant difference was observed in the levels of the P450arom transcripts among the quadrants. Therefore, the mean of values from the four quadrants was calculated for further comparisons. Total transcript levels were similar to those of the Q2-Q4 quadrants of the other breast cancer patients but were significantly higher (Fig. 4) in comparison with cancer-free subjects. Again, transcripts with promoter II-specific and exon I.3-specific 5′-termini were present at higher levels in comparison with those with exon I.4-specific 5′-termini (data not shown).

The data were further analyzed to observe if there was a difference in the expression of CYP19 gene transcripts in terms of age. Five of the breast cancer patients (Table 1) were under 50 yr of age. There were no significant differences in either total transcript levels or with respect to any of the 5′-termini studied between the over-50 and under-50 groups. Likewise, there seemed to be no significant difference in CYP19 gene transcript levels between the younger and older women undergoing reduction mammoplasty, but because of

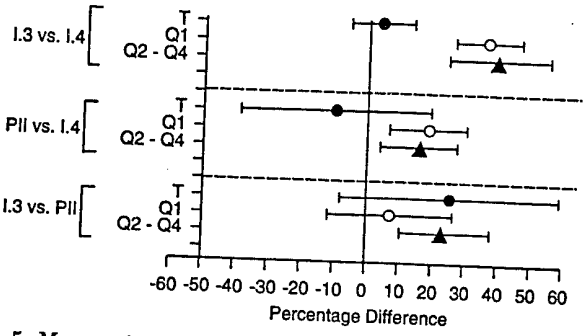


FIG. 5. Mean ± 95% confidence interval of percentage difference in expression level of pairs of transcripts by site. Under the normality assumption, the difference is considered significant at the level of alpha = 0.05 if the 95% confidence interval does not cross the zero line. Abbreviations are as described in the legend to Fig. 3.

the small number of samples, the possibility of age-related effects should not be ruled out. Additionally, because most of our patients were black, it was not possible to determine a role of ethnic origin on CYP19 gene transcripts levels.

## Discussion

Consistent with the notion that breast tumors elaborate factors that stimulate local expression of aromatase, is our observation that transcript expression in the quadrant that contains the tumor is greater than that in the remaining three quadrants. This is consistent with the findings of a number of studies that show that either aromatase activity (9, 31, 33) or expression (35) is highest in adipose tissue proximal to a tumor, as compared with tissues distal to a tumor. Only one published study (34) has, so far, failed to show such a distribution. However, the present study shows, for the first time, that breast adipose tissue from cancer-free individuals has a lower level of aromatase expression than adipose tissue from cancer patients, whether it be from the quadrant that contained the tumor or from the remaining three quadrants derived from the same breast. Thus, it seems that, within the tumor-containing breast, there is a gradient of aromatase-stimulating activity emanating from the tumor site. Another difference between the cancer patients and the cancer-free individuals relates to age, namely, that the latter are a younger group than the former. Earlier studies (3, 5) have shown an increase of aromatase activity and expression with age in other body sites, *e.g.* buttock, thigh, and abdomen. However, no such link has been established in breast adipose tissue. In the present study, there was no detectable difference in aromatase expression among women of different ages in either group. So we conclude that age is unlikely to be a major factor influencing the results reported here, although considering the small number of samples, the possibility of an effect of age on breast aromatase expression can not be ruled out.

To gain insight into the factors that stimulate aromatase expression in the breast tissue of individuals with breast cancer, in the present study we have examined the distribution of the various 5′-termini-specific transcripts in samples of breast adipose tissue removed from cancer-free individuals and individuals diagnosed as having breast cancer.

In breast adipose tissue samples from cancer-free individuals, we found that promoter I.4-specific transcripts were the most numerous, comprising at least 50% of the total P450arom transcripts, whereas promoter I.3- and promoter II-specific transcripts together made up the remaining 50% (Fig. 3). This is consistent with aromatase expression in adipose tissue of cancer-free individuals being regulated mainly by class I cytokines. IL-6, IL-11, and LIF all have been shown to be elaborated by adipose stromal cells in culture (38). Thus, in normal human adipose tissue, it seems that aromatase expression is under local control by such factors via paracrine and autocrine mechanisms. The issue, as to whether breast adipose tissue obtained as a consequence of reduction mammoplasty should be considered normal, is unclear at this time because this procedure is most commonly undertaken as a result of symptomatic macromastia. Nevertheless, in the absence of any evidence to the contrary, for the purposes of this study, such tissue is considered normal in the sense that it is derived from individuals who are cancer-free.

Of considerable surprise to us, therefore, was the observation that the increase in total P450arom expression that occurs in breast adipose tissue of patients with cancer was accompanied by only a small increase in promoter I.4-driven expression, but rather, was largely the result of an increase in expression from promoter II and promoter I.3 (Fig. 3). This was true not only in the quadrant that contained the tumor but in the other breast quadrants as well (Fig. 3). This conclusion is illustrated diagrammatically in Fig. 6. A similar result was briefly mentioned in reference (43) as a small study employing RT-PCR of five patients, in which three showed such a reversal. However, in that report, the methodology was not validated by the use of internal standards to control for variations in amplification.

Schor *et al.* (44) has reported that breast adipose tissue from patients with cancer has intralobular fibroblasts with a fetal fibroblast phenotype, whereas tissues obtained from disease-free patients do not have this type of fibroblast (44). These fetal-like fibroblasts also were present in histologically normal adipose tissue adjacent to the carcinoma. Therefore, it seems that the presence of a breast tumor imposes several changes in the phenotype of the surrounding mesenchymal cells, of which aromatase expression is only one.

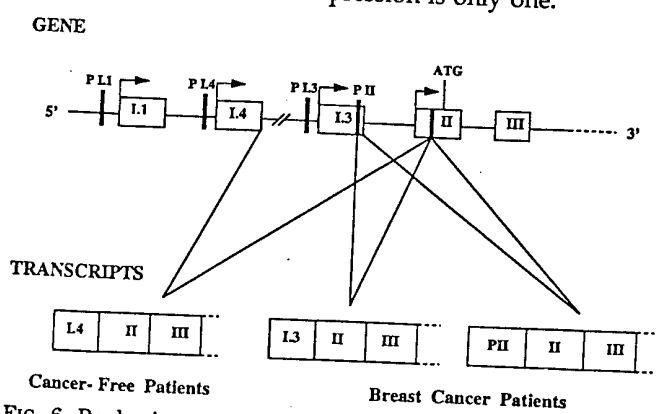


FIG. 6. Predominant exon-specific transcripts of the CYP19 gene in breast adipose tissue of cancer-free individuals and breast cancer patients, and their origin by alternative splicing.

These results imply that, in the presence of a tumor, factors that stimulate aromatase expression via promoter I.4 are not primarily responsible for the increase in expression in surrounding adipose fibroblasts, but rather some other influence, stimulating expression via promoter II and I.3, is dominant. In adipose stromal cells in culture, expression from promoters II and I.3 is stimulated by dibutyryl cAMP (27). Moreover this action of cAMP analogs is dramatically potentiated by phorbol esters (27). Thus, it would appear that in the presence of a tumor, P450arom expression is driven primarily by a factor or factors that stimulate adenylyl cyclase, perhaps in conjunction with protein kinase C activation. This factor or factors are presumably produced either by the tumor cells themselves or recruited into the tumor site as a consequence of an inflammatory response. Clearly, the challenge for the future will be to determine the nature of this factor or factors. This is important not only to further our understanding of the relationship between breast cancer and estrogen biosynthesis in adipose, but additionally, delineation of this signaling pathway may lead to the development of inhibitors of this pathway that would, therefore, inhibit aromatase expression specifically in adipose tissue of individuals with breast cancer. This may, in turn, lead to the development of novel therapies for the endocrine management of breast cancer that specifically eliminate estrogen produced locally at the tumor site.

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# Distribution of Aromatase P450 Transcripts and Adipose Fibroblasts in the Human Breast*

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**ABSTRACT**

The fibroblast component of adipose tissue is the primary extragladular site of aromatase P450 (P450arom) expression, which is responsible for the conversion of $C_{19}$ steroids to estrogens. Previously, we have shown positive correlations between the level of P450arom transcripts and the ratio of fibroblasts to mature adipocytes in adipose tissue samples proximal to breast tumors. The present study was conducted to determine the distribution of P450arom messenger ribonucleic acid (RNA) levels and fibroblast to adipocyte ratios in disease-free breasts of premenopausal women. Adipose tissue samples were collected from 3 regions (outer, upper, and inner) of both breasts of 13 women undergoing reduction mammoplasty. The histological composition of adipose tissue (n = 12) was determined by morphometry using a computerized image analysis program. We used a competitive RT-PCR method employing rat P450arom complementary RNA as an internal standard to quantify adipose P450arom transcripts in tissue total RNA samples (n = 11). Overall, 67% of the highest fibroblast to adipocyte ratios and 64% of the highest P450arom transcript levels were detected in an outer breast region, whereas in only 1 patient were the highest values detected in an inner region. Parametric ANOVA showed significant differences between the fibroblast content of the regions [p(F) = 0.037]. This distribution pattern directly correlates with the most common or the least common sites of carcinoma in the breast, the outer and inner regions, respectively. Moreover, a direct relationship was demonstrated between adipose fibroblasts and P450arom transcripts within the breast, in that regions with the highest fibroblast to adipocyte ratios contained the highest P450arom transcript levels (by ANOVA of contrast variables, $P = 0.0009$). These results suggest that, similar to our previous findings in the breast bearing a tumor, adipose tissue aromatase expression in the disease-free breast is determined by the local ratio of fibroblasts to adipocytes. (*J Clin Endocrinol Metab* **81:** 1273–1277, 1996)

THE CONVERSION of $C_{19}$ steroids to estrogens by aromatase P450 (P450arom) takes place in a number of human cells, *e.g.* the ovarian granulosa cell (1), the placental syncytiotrophoblast (2), and the adipose fibroblast (3, 4). Aromatase expression does not occur in mature adipocytes, but, rather, in the fibroblasts that are dispersed among the adipocytes (3, 4). Adipose fibroblasts are believed to be the preadipocytes that give rise to mature adipocytes in human adipose tissue. Aromatase expression in adipose tissue has long been implicated in the pathophysiology of breast cancer growth (5–8). Potent aromatase inhibitors have recently been widely used in the treatment of postmenopausal breast cancer (9–11). Peripheral estrogen production in whole body adipose tissue may be enough to effectively increase circulating levels of estrogens (5). Estrogen produced by adipose tissue within the breast also may act locally to promote the growth of breast tumors; O'Neill and co-workers (7) demonstrated that the breast quadrant displaying the highest level of aromatase activity was consistently involved with tumor. Furthermore, we found the highest levels of P450arom transcripts in fat from the quadrants bearing tumors in 69% of the cases (8). In the same study, the quadrants containing the highest fibroblast to adipocyte ratios had the highest P450arom levels.

The present study was conducted to ascertain the distributions of adipose fibroblast content and P450arom transcript levels in tumor-free breasts of women undergoing reduction mammoplasty. We previously demonstrated that both aromatase activity (3) and P450arom transcript levels (4) primarily reside within the fibroblast fraction of the adipose tissue, and we observed a positive correlation between fibroblast to adipocyte proportions and P450arom transcript levels in breast adipose tissue from mastectomy specimens bearing tumors (8). However, the presence of a tumor in breast adipose tissue in this instance may have influenced the distribution of P450arom expression in the breast. Therefore, the present study used reduction mammoplasty samples to investigate the distribution patterns of fibroblasts and P450arom transcript levels in the disease-free breasts of premenopausal women. Additionally, we investigated the possible effects of side (left *vs.* right) or position (outer, upper, or inner) on these distribution patterns.

## Materials and Methods

### *Tissue acquisition and processing*

Breast fat was obtained at the time of surgery from both breasts of 13 women undergoing reduction mammoplasty for symptomatic macromastia (6 samples/patient). Written consent was obtained be-

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fore surgery, including a consent form and protocol approved by the Institutional Review Board for Human Research of the University of Texas Southwestern Medical Center. Whole breast adipose tissue samples were frozen in liquid nitrogen and stored at −70 C.

Adipose tissue samples were obtained from the midportions of the outer and inner breast halves. A third sample was also obtained from the middle of the upper breast fat, which practically is the midpoint between the outer and inner samples above the nipple. This mode of sampling was dictated by the technique of the reduction mammoplasty operation.

### *Human ribonucleic acid (RNA) isolation and rat P450arom complementary RNA (cRNA) synthesis*

Human total RNA was isolated from frozen tissue of 11 women by the guanidinium thiocyanate-cesium chloride method (12). Rat P450arom cRNA was synthesized as previously described (4, 8).

### *Quantitative PCR after RT-PCR and hybridization*

RT-PCR using synthesized oligonucleotides as primers and radiolabeled probes was performed as previously described (4, 8). Briefly, an antisense oligonucleotide complementary to coding exon IV was used for primer extension. The created single stranded P450arom complementary DNA template was then amplified by PCR using the same antisense oligonucleotide and a sense oligonucleotide identical to a sequence in coding exon II. As an internal standard, 1 pg of a homologous rat P450arom cRNA (4, 8) was reverse transcribed and coamplified in each human breast total RNA sample to control and correct for the differences in amplification efficiency between these samples. The antisense and sense primers were designed as such to recognize identical sequences in both human and rat complementary DNAs. Amplified products were divided in half and transferred to separate membranes. Each membrane was hybridized with a species-specific labeled oligonucleotide probe designed to recognize the heterologous midportion of the amplified human or rat product. An AMBIS image acquisition and analysis system (AMBIS, Inc., San Diego, CA) was used to quantify radioactivity on each membrane. As each sample initially contained an equal amount of rat cRNA, the ratio of human to rat amplification product obtained for the P450arom messenger RNA (mRNA) level in each adipose tissue sample was reported as an arbitrary unit.

### *Histology*

Representative sections were obtained from each breast region (n = 12) using the same frozen adipose tissue fragments, parts of which were previously used for RNA isolation. Sections (3 μm thick) of paraffin-embedded tissue were stained with hematoxylin-eosin. Adipocytes, fibroblasts, acellular stroma, ductal epithelium, and endothelium-lined space components were quantified by a computer-assisted image processing program (Image 1.51, NIH, Bethesda, MD) and expressed as percentages of the total image in any particular field.

### *Statistical analysis*

Parametric repeated measures ANOVA followed by Newman-Keuls multiple comparisons test were used to compare fibroblast proportions and P450arom transcript levels between regions of individual breasts. Multivariate ANOVA for repeated measures and ANOVA of contrast variables were used to test the correlation between fibroblast proportions and P450arom levels. Simple linear regression analysis was used in establishing the standard curve.

## Results

### *Standard curve (Fig. 1)*

Initially, we established a range of starting amounts of human breast adipose total RNA (2–16 μg) that yielded linear increases in amplification product in the presence of a constant amount of coamplified rat P450arom cRNA (Fig. 1). The arbitrary units for P450arom mRNA levels were plotted against the initial breast adipose tissue total RNA quantities to create a standard simple regression line (r = 0.987; $P < 0.0001$). The samples were amplified in duplicate, and the mean value for each duplicate was reported as the P450arom transcript level for each sample. Individual values of the standard curve experiment remained within ±15–25% of the mean. This experiment was repeated twice with similar results. Using individual values instead of means of duplicates did not change the precision of this assay. Subsequent experiments employed 10 μg breast adipose RNA and 20 cycles of amplification, because under these conditions the intensity of the radioactivity on each slot fell within the linear range of increase in the human amplification product.

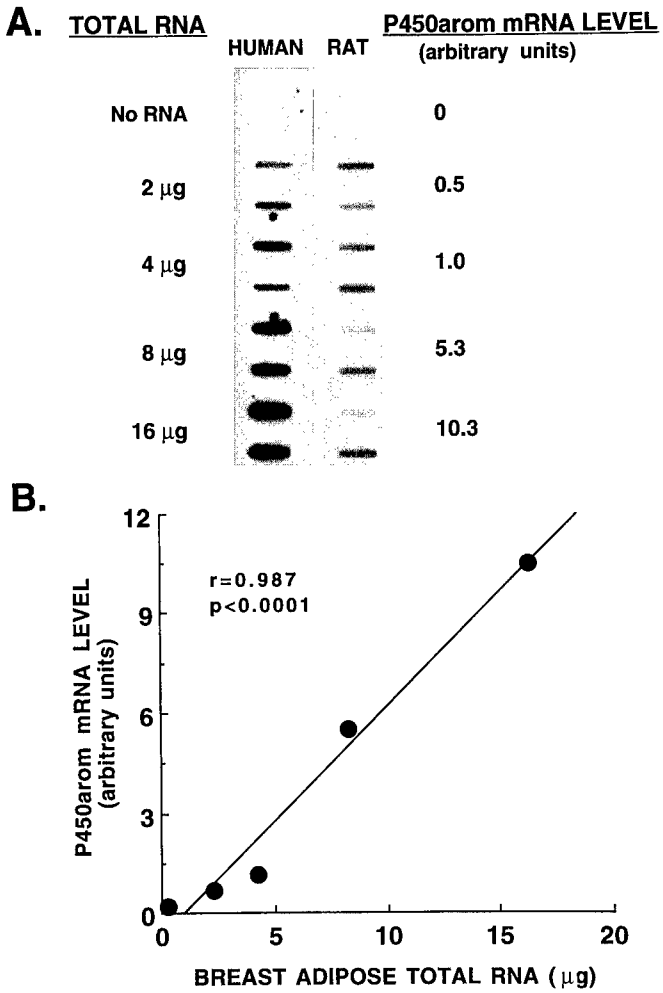


FIG. 1. Standard curve, revealing simple linear relationship between increasing amounts of total breast adipose tissue RNA and corresponding arbitrary units for P450arom transcript levels. A, Coamplification of increasing amounts of breast adipose tissue total RNA and a constant quantity of rat P450arom cRNA internal standard (1 pg). Samples were amplified in duplicate. B, A linear increase in breast P450arom levels (ratios of human to rat amplification products) was observed with increasing amounts of breast adipose tissue RNA. Statistical analysis was performed using simple linear regression with a coefficient of determination of 0.987 at a level of significance of $\alpha = 0.05$ and $P < 0.0001$.

*P450arom transcript levels in breast regions (Fig. 2 and Table 1)*

Figure 2 illustrates a representative experiment for 1 of the 11 patients. Eleven such separate experiments were performed, and P450arom transcript levels are listed in Table 1. P450arom transcript levels from 6 breast regions (3 on each side) were compared for each woman. P450arom transcripts were detected in all samples by autoradiography and the AMBIS Radioanalytic Imaging System. Parametric repeated measures ANOVA revealed a linear trend, with the highest levels found in the outer region followed by the upper region, and the lowest levels found in the inner region; however, this relationship did not reach statistical significance [p(F) = 0.110]. In 64% of the women, the highest levels were localized to an outer breast region, whereas in only 1 patient was the highest level found in an inner region. This distribution followed the same order as the fibroblast to adipocyte ratio distribution among the breast regions (see below).

*Distribution of fibroblasts in breast regions (Fig. 3 and Table 2)*

Sections of breast regions from 12 women revealed marked differences in the ratio of fibroblasts to adipocytes (1%-15%;

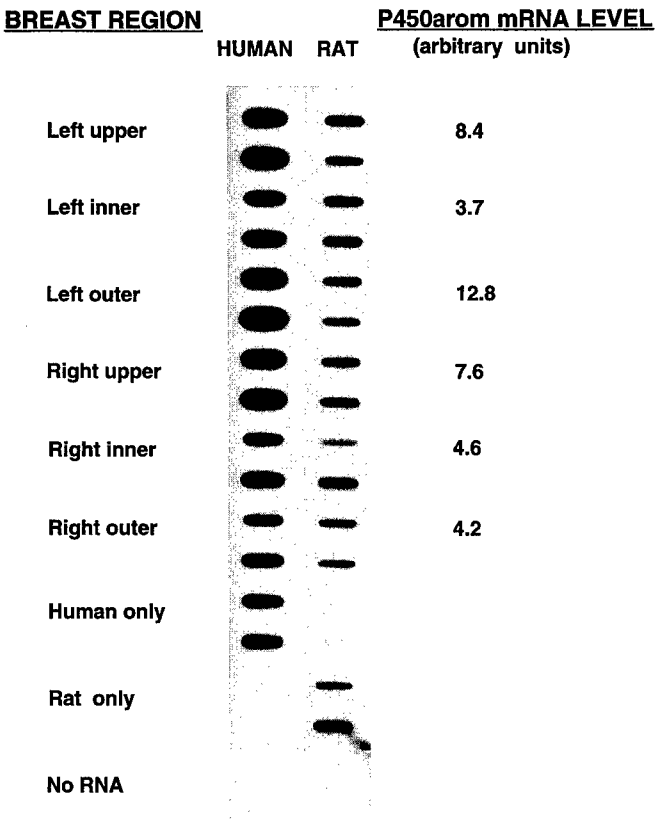


FIG. 2. A representative slot blot autoradiograph from one patient is shown. We compared P450arom transcript levels in breast fat using 10 μg total RNA from each quadrant. Each sample was assayed in duplicate (variation in duplicate samples ranged from 2–8%), and radioactivity on the blotting membranes was quantified by an AMBIS radioimaging system. A corrected AMBIS reading was calculated and recorded as an arbitrary unit for the P450arom transcript level for each quadrant, as described in Fig. 1.

**TABLE 1.** Adipose tissue P450arom transcript levels in breast regions

| Patient No. | Left | | | Right | | |
|---|---|---|---|---|---|---|
| | Inner | Upper | Outer | Inner | Upper | Outer |
| 1 | 0.21 | 0.28 | 0.12 | 0.21 | 0.10 | 0.07 |
| 2 | 1.31 | 1.80 | 2.23 | 1.23 | 2.47 | 1.74 |
| 3 | 0.13 | 0.13 | 0.18 | 0.11 | 0.14 | 0.22 |
| 4 | 2.76 | 2.80 | 2.29 | 3.85 | 1.98 | 2.23 |
| 5 | 1.01 | 0.80 | 0.33 | 1.58 | 1.24 | 2.54 |
| 6 | 0.07 | 0.03 | 0.08 | 0.05 | 0.01 | 0.07 |
| 7 | 3.67 | 8.40 | 12.80 | 4.60 | 7.60 | 4.20 |
| 8 | 0.96 | 1.60 | 1.01 | 1.07 | 0.77 | 1.90 |
| 9 | 1.30 | 4.60 | 4.70 | 2.30 | 3.30 | 4.84 |
| 10 | 1.25 | 3.78 | 2.03 | 3.46 | 3.40 | 3.08 |
| 11 | 1.20 | 1.89 | 1.03 | 1.87 | 1.31 | 1.95 |

Fig. 3). Mature adipocytes comprised the remainder of the sections. Glandular epithelium, vascular endothelial cells, and acellular stroma were noted in some samples in negligible proportions. Fibroblast to adipocyte ratios are listed in Table 2. Sixty-seven percent of the highest ratios were found in an outer breast region, whereas only 1 patient had the highest fibroblast to adipocyte ratio in an inner region. Parametric repeated measures ANOVA revealed significant differences between regions of individual breasts [p(F) = 0.037]. Newman-Keuls multiple comparisons test showed significant differences between the outer and inner or outer and upper regions ($P < 0.5$), but no significant difference was found between upper and inner regions.

P450arom transcript levels and fibroblast content were determined in the identical breast adipose tissue samples of 10 women. There was a statistically significant direct relationship between the distributions of fibroblasts and P450arom mRNA levels in the breast. In other words, proportions of fibroblasts in the 6 breast regions of a woman increased in the same order as the P450arom transcript levels. Multivariate ANOVA comparing regional fibroblast to adipocyte ratios with regional P450arom mRNA levels revealed a direct relationship ($P = 0.023$). Additionally, ANOVA of contrast variables showed that regional fibroblast to adipocyte ratios in the breast increased, with significant differences between each other when they were arranged to match regions with increasing order of P450arom mRNA levels ($P = 0.0009$). No influence of side (left *vs.* right) was noted on the distribution of P450arom transcripts or fibroblasts.

## Discussion

Adipose tissue has a similar uniform histological appearance in various parts of the human body; however, evidence from several laboratories, including ours, suggests that the endocrinological behavior of this tissue may be different in the breast, abdomen, buttock, and thigh of a woman (13–15). Differences in adipose tissue aromatase expression at various body sites of women have been well characterized. Aromatase expression in adipose tissue samples of women was highest in the buttock region, followed by that in the thigh, and the lowest in the abdomen (13). Thus far, breast adipose tissue aromatase expression has not been directly compared to that at other body sites. We found that different promoters are responsible for aromatase expression in adipose tissue in

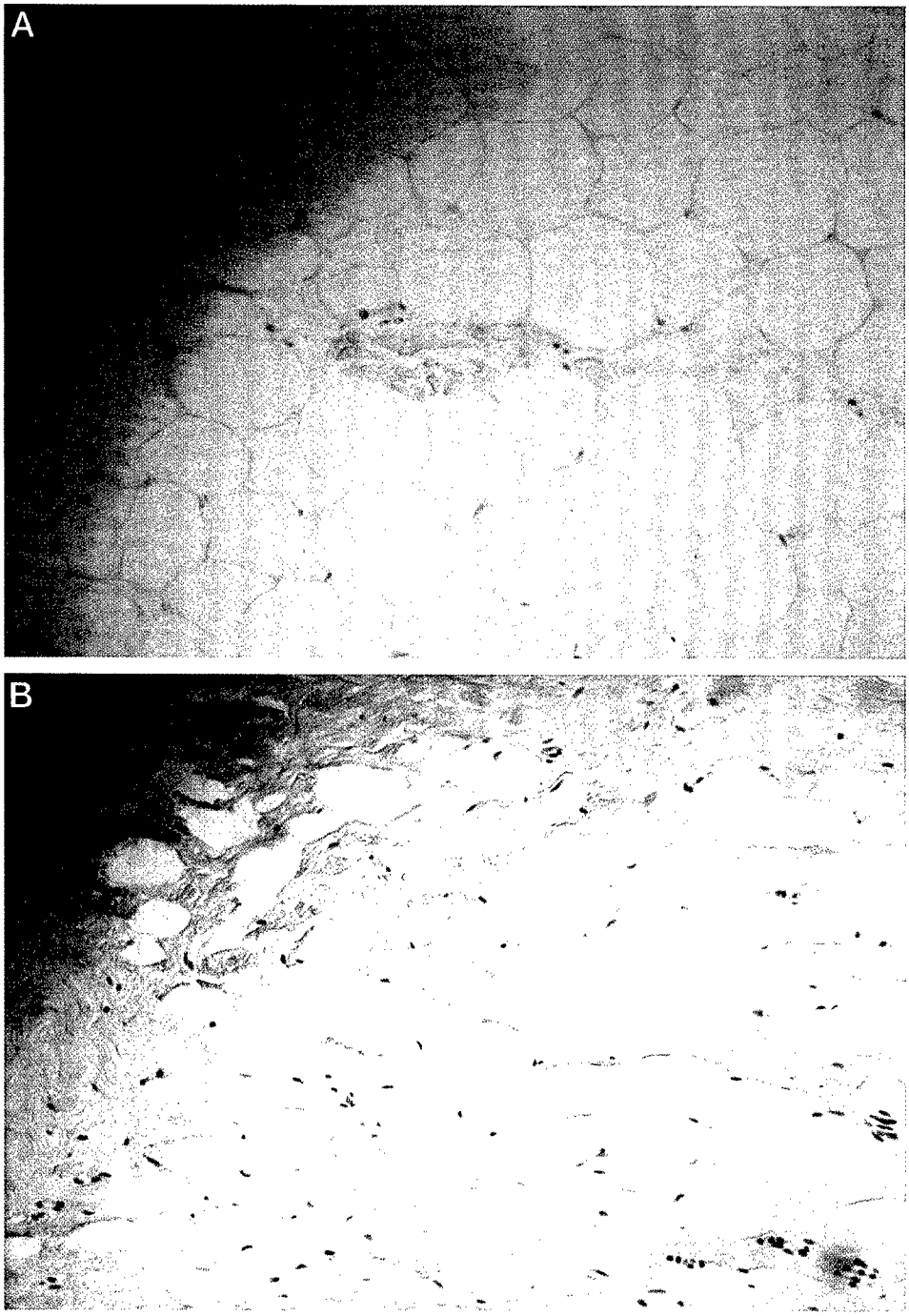


FIG. 3. A, A representative section of the breast adipose tissue with low fibroblast content. Whereas mature adipocytes (large cells with cytoplasms distended by lipid material and peripheral nuclei) constitute the majority of this section, a few scattered fibroblasts dispersed between mature adipocytes are seen. A very low P450arom mRNA level (normalized to total RNA content) was detected in this sample. B, A breast fat specimen showing higher numbers of fibroblasts. Fibroblasts are oval or spindle-shaped and do not contain conspicuous lipid in their cytoplasms. A markedly higher level of P450arom mRNA was found in this sample. Hematoxylin and eosin stain; magnification, ×200. Note that handling and magnification for both sections are the same, whereas the sizes of the adipocytes vary. Tissue components were quantified by computerized morphometry. A positive correlation was found between the tissue fibroblast content and the P450arom transcript level in breast adipose tissue.

breast and lower thighs (16). This suggests that different signal transduction pathways are responsible for aromatase expression at these two sites. Finally, in contrast to variable expression in breast regions, the marked differences in aromatase expression in the abdomen, buttock, and thigh are not related to the fibroblast to adipocyte ratios at these body sites (our unpublished observations). This suggests that in contrast to breast regions, intrinsic differences in the specific aromatase expression among the fibroblasts from these other body sites, rather than fibroblast numbers, determine local estrogen biosynthesis.

The studied group of women undergoing reduction mammoplasty suffered symptomatic macromastia. The etiology of this condition is not known, and it is possible that breast aromatase expression in these women may be significantly different from that in women with smaller breasts. Therefore, our results should be viewed with a degree of caution when applied to the general population. Nevertheless, the subjects in this study represent a premenopausal group of women with cancer-free breasts, in contrast to our previous study group that was comprised of postmenopausal women with breast cancer (8). Theoretically, general anesthesia and its associated stress may give rise to elevated cortisol levels, which may effect aromatase expression in adipose tissue.

**TABLE 2.** Fibroblast to adipocyte ratios in breast regions

| Patient No. | Left | | | Right | | |
|---|---|---|---|---|---|---|
| | Inner | Upper | Outer | Inner | Upper | Outer |
| 1 | 0.03 | 0.15 | 0.12 | 0.09 | 0.05 | 0.05 |
| 2 | 0.02 | 0.06 | 0.10 | 0.04 | 0.05 | 0.06 |
| 3 | 0.12 | 0.11 | 0.13 | 0.04 | 0.07 | 0.11 |
| 4 | 0.12 | 0.02 | 0.05 | 0.12 | 0.04 | 0.06 |
| 5 | 0.06 | 0.04 | 0.09 | 0.10 | 0.10 | 0.05 |
| 6 | 0.05 | 0.03 | 0.11 | 0.04 | 0.03 | 0.06 |
| 7 | 0.05 | 0.15 | 0.05 | 0.06 | 0.09 | 0.15 |
| 8 | 0.10 | 0.06 | 0.08 | 0.09 | 0.05 | 0.12 |
| 9 | 0.03 | 0.04 | 0.01 | 0.02 | 0.02 | 0.07 |
| 10 | 0.03 | 0.05 | 0.06 | 0.10 | 0.04 | 0.10 |
| 11 | 0.05 | 0.04 | 0.06 | 0.07 | 0.10 | 0.12 |
| 12 | 0.04 | 0.03 | 0.10 | 0.07 | 0.05 | 0.11 |

However, pharmacological doses of glucocorticoids are capable of causing a detectable increase in aromatase activity of adipose fibroblasts after 3 h of treatment (17), whereas the samples for this study were collected within 1 h after initiation of general anesthesia. Therefore, an effect of general anesthesia on our results through this mechanism is unlikely. Finally, in this study, P450arom transcripts determined in breast adipose tissue are assumed to be translated into P450arom protein with aromatase activity, which gives rise to local estrogen biosynthesis. At least two published studies from this laboratory support this assumption. Significant levels of aromatase activity have been demonstrated in adipose tissue (3), and aromatase activity of adipose fibroblasts has been shown to be primarily regulated by changes in P450arom transcript levels (18).

One of every nine American women will develop breast cancer. More than two thirds of breast carcinomas develop in women over the age of 50 yr. The present study provides data of an associative nature and does not necessarily demonstrate a cause and effect relationship between local estrogen biosynthesis and the occurrence of breast cancer. However, it suggests that local factors in the breast may influence carcinogenesis. It has been hypothesized that 5–15 yr are required for a single malignant cell to develop into a clinically detectable breast cancer tissue mass. One may then assume that conditions that predispose a postmenopausal woman to develop breast cancer may preexist in her younger years. High local estrogen concentrations in the breast may predispose cancers initiated in these areas to develop more aggressively than those in others. Breast cancer occurs in the outer quadrants in 71% of the cases in a large series (19). The present study reveals that 64–67% of the highest P450arom transcript levels and adipose fibroblast content were also found in an outer breast region. On the other hand, we have previously shown in mastectomy specimens that the highest transcript levels were detected in regions bearing tumors regardless of the tumor location (8). Tumors secrete products (20), *e.g.* members of the interleukin-6 cytokine subfamily, that stimulate aromatase expression in the surrounding adipose tissue (21). Moreover, estradiol stimulates cytokine secretion by T-47D breast cancer cells (our unpublished observations). These data collectively suggest that tumors preferentially develop in breast regions with the highest aromatase expression. Once developed, tumor growth is further promoted by high local estrogen concentrations that are maintained by the positive feedback relationship between adipose tissue aromatase expression and tumor-derived cytokines.

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# Quantitative detection of alternatively spliced transcripts of the aromatase cytochrome P450 (*CYP19*) gene in aromatase-expressing human cells by competitive RT-PCR

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C19 steroids are converted to oestrogens in a number of tissues by a specific form of cytochrome P450, namely aromatase P450 (P450arom; the product of the *CYP19* gene). The human *CYP19* gene comprises nine coding exons, II–X. The tissue-specific expression is determined by the use of tissue-specific promoters, which give rise to P450arom transcripts with unique 5′-untranslated sequences. The majority of the transcripts present in the ovary contain promoter-II-specific sequences, while transcripts in the placenta contain exon I.1. Transcripts in adipose tissue possess exon I.3 and exon I.4. Also, the distribution of alternative transcripts in adipose stromal cells depends on the culture conditions. Therefore, a competitive RT-PCR method was designed to quantitatively detect alternatively spliced transcripts present in various tissues and cells maintained in different culture conditions. Specific synthetic transcripts with different 5′ termini (exon I.3, exon I.4 and promoter-II-specific sequences) and the coding region were used as internal standards. This competitive RT-PCR method was used to quantitatively detect three 5′ termini, i.e. promoter-II-specific sequence, exon I.3 and exon I.4, in transcripts in human adipose stromal cells and ovarian granulosa cells in primary culture. The quantity of total P450arom transcripts was judged by amplifying the coding region. We were also able to quantify rare transcripts which could not be detected previously by Northern analysis. 

***KEYWORDS:*** alternatively spliced transcripts, competitive RT-PCR, human, aromatase cytochrome P450 (*CYP19*) gene.

## INTRODUCTION

The biosynthesis of oestrogens from androgens is catalysed by an enzyme termed aromatase cytochrome P450 (P450arom; the product of the *CYP19* gene) which is localized in the endoplasmic reticulum of cells in which it is expressed.[1–5] This enzyme is responsible for binding the $C_{19}$ steroid substrate and catalysing the series of reactions leading to the formation of the phenolic A ring characteristic of oestrogens.[6–10]

In most vertebrate species examined, aromatase expression occurs in the gonads and in the brain. In the case of humans, the aromatization reaction occurs

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in a number of cells and tissues including ovarian granulosa cells,[11] testicular Sertoli,[12] Leydig cells,[13,14] placenta[2,3] and adipose tissue of both males and females,[15] and various sites of the brain including the hypothalamus,[16] amygdala and hippocampus.[17] However, the principal sites of oestrogen biosynthesis in the human female are the ovarian granulosa cells in pre-menopausal women, the placenta in pregnant women and adipose tissue in post-menopausal women. The physiological significance of oestrogen biosynthesis in human placenta and adipose tissue is unclear at this time; however, the latter has been implicated in a number of pathophysiologic conditions. Oestrogen biosynthesis by adipose tissue not only increases as a function of body weight but also as a function of age.[18,19] Further, there appears to be a relationship between oestrogen biosynthesis in adipose and several disease states such as post-menopausal endometrial and breast cancer.[20,21]

The human *CYP19* gene encoding aromatase cytochrome P450 has been cloned and spans more than 75 kilobases (kb) in size; however, the coding region is about 35 kb and contains nine exons (II–X). The heme-binding region is located on the last coding exon (X), and the translation initiation site is located in exon II. Upstream of exon II are located a number of untranslated exons which are spliced into the 5′ ends of transcripts in a tissue-specific fashion.[22] For this reason, specific 5′ termini are found in the transcripts encoding P450arom in different tissues. All of the sequences are spliced onto exon II at the same 3′ splice junction, upstream of the start of translation, and thus the coding region and the protein are the same regardless of the tissue-site of expression. The majority of the transcripts present in the ovary contain sequences specific for promoter II, whereas transcripts in the placenta contain sequences specific for exon I.1. Adipose tissue expresses two species of transcripts, containing exon I.3- and I.4-specific sequences.[23] Also, the distribution of alternative transcripts in adipose stromal cells[23] depends on their culture conditions. Thus, the cytochrome P450arom gene encodes a number of alternatively spliced transcripts in various tissues regulated by tissue-specific promoters. In order to comprehend the regulation of the *CYP19* gene in any given tissue, it is very important to have a quantitative estimation of the various transcripts containing the different 5′ termini present in that particular tissue. The RACE (rapid amplification of cDNA ends) procedure was utilized previously to amplify alternative 5′ termini sequences from P450 arom transcripts.[23] However, this method may not amplify different transcripts with equal efficiency, and thus may not yield a true quantitative distribution of the various 5′ termini in *CYP19* gene transcripts in a particular cell or tissue. Northern analysis can detect only abundantly expressed transcripts. Therefore, considering the complexity of the expression of alternatively spliced transcripts of the *CYP19* gene, it became essential to design a sensitive method to detect the 5′ termini of various transcripts.

Here, we describe a competitive RT-PCR method to quantify three major 5′ termini of *CYP19* gene transcripts, i.e. promoter-II-specific and exon I.3- and exon I.4-specific. Using this method we were able to quantitatively detect multiple transcripts present in primary cultures of human adipose stromal cells and ovarian granulosa cells grown in different culture conditions. Also, for the first time, we report analysis of the alternative transcripts present in cultured adipose stromal cells treated with dexamethasone and the cytokine IL-11. Most importantly, this method is also applicable to quantitative analysis of alternatively spliced transcripts of other genes in which this form of regulation of expression is utilized.

## MATERIALS AND METHODS

### Internal standards

Figure 1 schematically depicts the principle and steps involved in generating internal standards which were used as competitors for RT-PCR of human P450arom transcripts. To prepare internal standards, three PCR products (AP-PII, AP-I.3 and AP-I.4) were amplified from partial cDNA clones (RACE clones[23]) specific for promoter-II-specific sequence, exon I.3 and exon I.4, respectively. RT-1, RT-2, RT-3 (sense oligos specific for each untranslated exon) and RT-4 (antisense oligo for exon II) were used as primers, and their sequences are given in Table 1. Simultaneously, one PCR product (AP-Cod) was amplified from a cytochrome P450arom cDNA clone[24] using the sense primer (RT-5) from the non-coding region of exon II and the antisense primer (RT-6) from exon IV. PCRs were set up as described in the detailed protocol of RT-PCR (see following paragraph), except [$\alpha$-$^{32}$P] dCTP was not included, and were performed on a Gene Amp PCR system 9600 (Perkin-Elmer). Reaction conditions were as follows: denaturing temperature 93°C for 1 min, annealing temperature 41°C for 1 min, extension temperature 72°C for 1 min, total cycles 30. By combining one of each of the AP-PII, AP-I.3, AP-I.4 (PCR products) with AP-Cod (PCR product), PCR was used to amplify three different sequences containing alternative 5′ termini, i.e. promoter-II-specific sequence, exon I.3 or exon I.4. The antisense primer (RT-13, Table 2) contains a 27 bp random AT rich

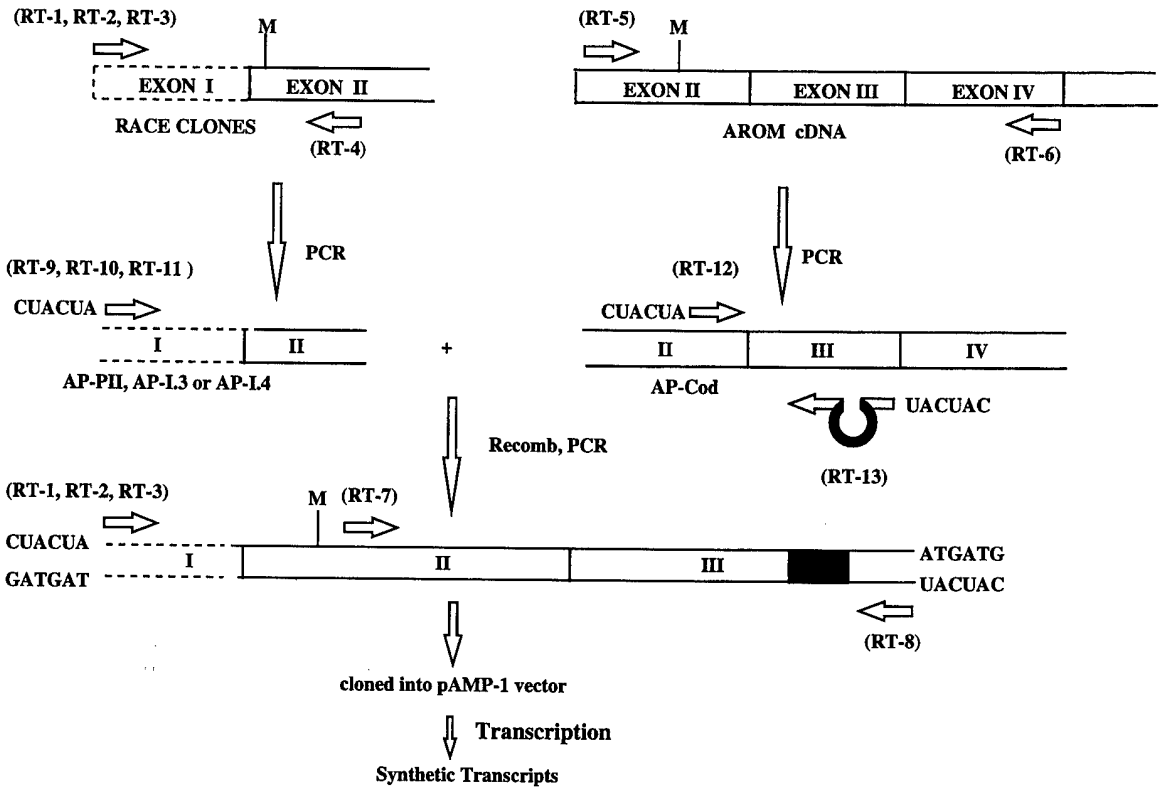


**Fig. 1.** Schematic diagram of the synthesis of internal standard transcripts. Dashed lines depicts the variable region of clones. All numbers in parentheses represent the oligo number (for sequences see Tables 1 and 2). Black region in oligo RT-13 and final PCR product represents the 27 bp random AT-rich insert sequence (loop).

**Table 1.** Oligonucleotides used as primers for PCR amplifications

| | |
|---|---|
| RT-1 | 5′ end sense oligo from exon I.4<br>5′ GTG ACC AAC TGG AGC CTG 3′ |
| RT-2 | 5′ end sense oligo from exon I.3<br>5′ GAT AAG GTT CTA TCA GAC C 3′ |
| RT-3 | 5′ end sense oligo from promoter-II-specific sequence<br>5′ GCA ACA GGA GCT ATA GAT 3′ |
| RT-4 | 3′ end antisense oligo from coding exon II<br>5′ CAG GCA CGA TGC TGG TGA TG 3′ |
| RT-5 | 5′ end sense oligo from untranslated region of exon II<br>5′ TCT GAG GTC AAG GAA CAC 3′ |
| RT-6 | 3′ end antisense oligo from exon IV<br>5′ TTG TTG TTA AAT ATG ATG C 3′ |
| RT-7 | 5′ end sense oligo from coding exon II<br>5′ TTG GAA ATG CTG AAC CCG AT 3′ |
| RT-8 | 3′ end antisense oligo from exon III<br>5′ CAG GAA TCT GCC GTG GGG AT 3′ |
| GAPDH1 | 5′ end sense oligo from GAPDH gene<br>5′ CGG AGT CAA CGG ATT TGG TCG TAT 3′ |
| GAPDH2 | 3′ end antisense oligo from GAPDH gene<br>5′ AGC CTT CTC CAT GGT GGT GAA GAC 3′ |

region (loop[25]) and was designed to include a sequence specific to exon III on either side of the 27 bp loop region (Fig. 1). Sense primers (RT-9, RT-10, RT-11) were 5′ termini specific. Primers used in these reactions are listed in Table 2. A similar reaction was set up for amplifying part of the coding region (Exon II–Exon III) using primers RT-12 and RT-13 (Table 2) and a P450arom cDNA clone[24] as a template. CUA tails in the sense primers (5′ end primers, RT-9, RT-10, RT-11, RT-12) and a CAU tail in the antisense primer (3′ end primer, RT-13) were incorporated according to the CloneAmp System instructions (Gibco-BRL). The PCR products were then cloned into the pAMP-1 vector. These constructs were transcribed using SP6 polymerase according to the Promega Kit (Madison, WI, USA). Thus four internal standard transcripts were synthesized, each with the 27 bp random AT rich loop. Three of them are specific for the three major 5′ termini of the *CYP19* gene, i.e. exon I.3, I.4 and promoter-II-specific sequences and one transcript specific for the coding region between exons II and III. A known copy number of these transcripts was added to each RT-PCR reaction containing target RNA from the various tissues studied.

**Table 2.** Oligonucleotides used as primers for PCR amplification of specific regions of alternate exons I, which were subsequently cloned into the vector

| | |
|---|---|
| RT-9 | 5′ end sense oligo from exon I.4<br>5′ CUA CUA CUA CUA GTA GAA CGT GAC CAA CTG 3′ |
| RT-10 | 5′ end sense oligo from exon I.3<br>5′ CUA CUA CUA CUA GAT AAG GTT CTA TCA GAC 3′ |
| RT-11 | 5′ end sense oligo from promoter-II-specific sequence<br>5′ CUA CUA CUA CUA GCA ACA GGA GCT ATA GAT 3′ |
| RT-12 | 5′ end sense oligo from coding exon II<br>5′ CUA CUA CUA CUA TTG GAA ATG CTG AAC CCG AT 3′ |
| RT-13 | 3′ end antisense oligo from exon III containing a 27 bp random AT rich insert<br>5′ CAU CAU CAU CAU CAC AGG AAT CTG CCG TGG GGA TGA GGG GTC CAA TTC AAA TAT TTA TAT TAA AAT TAT TTT AAA CCA TGC AGT AGC CAG 3′ |

Underlined nucleotides represent a 27 bp random AT rich sequence inserted in the oligonucleotide.

**Table 3.** Expected sizes of PCR amplified products

| Alternative Exon I | Target | Internal standard |
|---|---|---|
| Exon I.4–Exon III | 294 bp | 321 bp |
| Exon I.3–Exon III | 395 bp | 422 bp |
| Exon I.3 (truncate)–Exon III | 289 bp | |
| Exon II–Exon III (promoter-II-specific) | 305 bp | 332 bp |
| Exon II–Exon III (coding region) | 194 bp | 221 bp |
| GAPDH | 306 bp | |

## RT-PCR

RNA samples were treated with DNase I (Gibco-BRL) at room temperature for 15 min to remove DNA contamination from the RNA samples. DNase I was inactivated at 90°C for 5 min. Then RNA was denatured at 65°C for 2 min and annealed to random hexamers (Gibco-BRL) by allowing the samples to cool slowly to 37°C. To synthesize the complementary DNA (cDNA), the annealed RNA was reverse-transcribed using Superscript II (Gibco-BRL) at 42°C for 1 h in the presence of 166 μM of each dNTP, 50 mM Tris-HCl (pH 8·3), 75 mM KCL, 3 mM $MgCl_2$, 10 mM DTT and 5 U of RNasin (Promega). cDNA was amplified using the polymerase chain reaction in the presence of 200 μM of each dNTP, 4 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8·3, 0·001% (w/v) gelatin, 10 pmol of each primer, 0·3 μCi of [α-$^{32}$P] dCTP (Amersham) and 2 U of *Taq* Polymerase (Perkin-Elmer) in a total volume of 10 μl. RT-1, RT-2, RT-3 and RT-7 primers (Table 1), specific for exon I.4, exon I.3, promoter-II-specific sequence and coding exon II respectively, were used as 5′ end antisense primers. RT-8 primer (Table 1) specific for exon III was used as 3′ end antisense primer. PCR conditions were: denaturing at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 30 s, for 25 cycles and were performed on a Gene Amp PCR system 9600 (Perkin-Elmer). One quarter of the PCR products was analysed on 4% non-denaturing polyacrylamide gels. Labelled molecular weight marker (pBR322-Msp1-digested, New England Biolabs) was loaded simultaneously on the gel to assess the sizes of the amplified products. The molecular weight marker was end-labelled using 10 U of T4 polynucleotide kinase (Gibco-BRL) in the presence of 70 mM Tris-HCl (pH 7·6), 10 mM $MgCl_2$, 100 mM KCl, 1 mM of 2-mercaptoethanol and 30 μCi of [γ-$^{32}$P] ATP (Amersham). Gels were either autoradiographed with Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA) or scanned on a PhosphorImager (Molecular Dynamics, CA, USA) and quantitatively analysed using ImageQuant software. Expected sizes of PCR products are listed in Table 3.

To check the integrity of cDNA, the 'housekeeping' gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous marker. Primers were designed from the sequence deposited in Genbank (Table 1). cDNAs were amplified in the presence of 2 pmol of each primer, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8·3), 1·5 mM $MgCl_2$, 50 mM KCl, 0·001% (w/v) gelatin, 0·3 μCi of [α-$^{32}$P] dCTP (Amersham) and 2 U of *Taq* polymerase (Perkin-Elmer) at 94°C for 1 min and 72°C for 1 min for 22 cycles.

## RNA isolation

Total RNA was isolated from adipose stromal cells according to the described method with minor modifications.[26] Dishes of frozen confluent adipose stromal cells were scraped in guanidinium thiocyanate. Total RNA from granulosa cells was extracted as described.[27] Total RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water and then precipitated with the addition of ethanol and potassium acetate.[26]

## Cell culture

Subcutaneous adipose tissue was obtained from women at the time of reduction mammoplasty. Follicles were obtained from women undergoing hysterectomy or bilateral oophorectomy for benign gynecological disease. Written consent was given preoperatively for the use of these tissues using a consent form. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were isolated and maintained as primary cultures in Waymouth's enriched medium containing Nu Serum (15%, v/v) (Collaborative Research Inc.) as described previously.[28] Upon reaching confluence, the cells were placed in serum-free or FCS-containing (15%, v/v) Waymouth's enriched medium for 24 h. The cells were then treated with dexamethasone (250 nM) in medium containing 15% FCS for 48 h to maximally induce P450arom mRNA levels. For treatment with dexamethasone and IL-11, IL-11 (10 ng $ml^{-1}$) was added to the culture medium after dexamethasone treatment and cells were incubated for 24 h. Media were removed, and the cells were frozen at −70°C until used for RNA isolation. Human granulosa-lutein cells were obtained and cultured as previously described.[29] Cultured granulosa cells were treated with 10 μM forskolin for 48 h.

## RESULTS

Herein we describe a sensitive quantitative RT-PCR method to detect three major alternatively spliced transcripts of the *CYP19* gene in human adipose stromal cells and granulosa cells. By using this method, we were also able to amplify the 5′ termini of transcripts which were present in very low copy number in tissues in the presence of other abundantly expressed transcripts. Before discussing these results in detail, we will describe the standardization and validation of our RT-PCR method.

A number of experiments were performed to select one set of PCR conditions and reagent concentrations (oligonucleotides, dNTPs and $Mg^{2+}$) which efficiently amplified all three 5′ ends of alternatively spliced transcripts (data not shown). Comparative efficiency of PCR amplifications for the 5′ termini, i.e. promoter-II-specific sequence, exon I.3, exon I.4 and the coding region of the P450arom gene, were determined experimentally. For these experiments, we used only the internal standard transcripts (synthetic transcripts), because the only difference between synthetic transcripts and target sequences was the 27 bp random

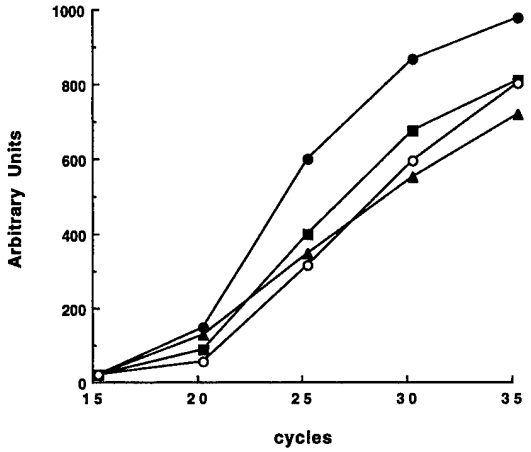


**Fig. 2.** The effect of the number of PCR cycles on amplification of synthetic transcripts (150 fg). Four reactions were set up, one for each of the synthetic transcripts. Aliquots were collected after the indicated number of cycles and subjected to gel electrophoresis and quantification by means of a phosphorimager. ▲, cod; ●, PII; ■ I.3; ○, I.4.

AT rich sequence inserted into the synthetic transcripts. Our PCR primers do not include that sequence, and are similar for target and synthetic transcripts. We preferred to use synthetic transcripts in comparison to amplified DNA fragments because we wanted to eliminate the differences caused by reverse transcription. Thus we mimicked all conditions for synthetic transcripts (internal standard) as for target transcripts. Therefore, amplification of the synthetic transcripts will reflect the efficiency of amplification of the corresponding unknown target transcripts. Experiments were designed to address each of the following issues:

(1) The quantitative nature of PCR has previously been questioned.[30,31] This conclusion was largely based on techniques which involved a high number of PCR cycles. To introduce more sensitive methods for detecting PCR products, submaximal numbers of cycles have been used which lie in the linear range of amplification. Amplification of the 5′ terminus of each transcript was tested after 15, 20, 25, 30 and 35 cycles by using the appropriate specific primers (Fig. 2). Twenty-five cycles were found to be the most appropriate because amplification under these conditions is within the linear range and is also submaximal. All experiments described hereafter employed amplification for 25 cycles.

(2) The amplification efficiency of transcripts containing three alternate 5′ termini (e.g. exon I.3, I.4 and promoter-II-specific) and the coding region may not be equal. To answer this question, equal quantities of all four synthetic transcripts were reverse transcribed according to the protocol described in

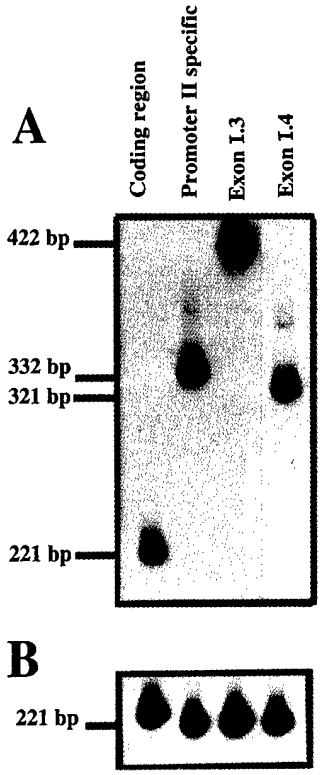


**Fig. 3.** Amplification of synthetic transcripts. (A) Each transcript was amplified using the appropriate specific primers as indicated. (B) Each transcript was amplified using coding-region-specific primers.

Materials and Methods and amplified by PCR using their corresponding primers (Table 1). Results are shown in Fig. 3. As expected, amplified bands were of expected size and of almost the same intensity (Fig. 3A). Equal quantities of all transcripts used were confirmed by amplification of the coding region (exon II–exon III) specific sequence which was common to all transcripts (Fig. 3B).

(3) Specific amplification of each 5′ terminus from the mixture of P450arom transcripts containing alternate 5′ termini may not be possible. To ensure that the products generated in the RT-PCR method were specific for the desired 5′ termini, we set up four PCRs using the cDNA of one synthetic transcript (internal standard), e.g. exon I.3-specific transcript, and four sets of primers for exon I.3, I.4, promoter-II-specific sequence and the coding region in each reaction (Fig. 4). Similar reactions were also set up for the cDNAs of the other three synthetic transcripts, i.e. for exon I.4, promoter-II-specific and coding region. As was expected, no cross-reaction (amplification) was detected, not even weak amplification after very long exposure. Thus, each synthetic transcript specific for one 5′ terminus was only amplified by primers specific for that sequence and for the coding region (Fig. 4).

(4) The amplification efficiencies of each transcript in the presence of the other alternatively spliced transcripts may not be equal. One picogram of each of the three synthetic transcripts, exon I.3, exon I.4 and promoter-II-specific, were mixed together. To approximate *in vivo* conditions, 50 ng of sheep kidney (a tissue lacking *CYP19* gene expression) RNA was added. The mixture was reverse transcribed, divided into four tubes and amplified by using the four sets of primers (Fig. 5). Amplification efficiencies of exon I.3, exon I.4 and promoter-II-specific sequences were almost similar. As was expected, amplification of the coding region was approximately three times higher than of other regions because the coding region is common in all synthetic transcripts.

(5) The most important question was: when varying quantities of alternatively spliced transcripts are present, will the amplification efficiency of the 5′ terminus of any one transcript be affected? To answer this question, we designed nine experiments (Table 4). Various quantities of each synthetic transcript were mixed with a constant amount of the other two transcripts resulting in nine different combinations. Sheep kidney RNA (50 ng) was added in each experiment to approximate an *in vivo* situation. Results are shown only for two experiments using varying concentrations of promoter-II-specific transcripts (Fig. 6). Similar experiments were performed with various concentrations of exon I.3 and exon I.4-specific transcripts (data not shown). We concluded that the amplification efficiency of the 5′ terminus of one transcript as a function of its concentration is not affected by the presence of different concentrations of other alternate transcripts (Fig. 6).

For standardization purposes, we used synthetic transcripts (internal standard) alone because they reflect the amplification efficiency of target sequences. When these synthetic transcripts were mixed with unknown target RNA, they should compete so that by comparing amplification of known quantities of internal standard, quantities of unknown transcripts can be calculated. Our goal was to quantitatively detect the 5′ termini of the several alternatively spliced transcripts from cells grown under different culture conditions as well as from tissues. Thus, we mixed three different quantities of internal standard transcript (coding-region specific) with the same quantity of RNA from adipose stromal cells grown in the absence of serum. As was expected, two amplified fragments were observed (Fig. 7). Internal standard cDNA amplification (221 bp) was competing with target cDNA amplification (194 bp). Identical experiments were performed using internal standard transcripts specific for exon I.4, exon I.3 and promoter-II-specific sequences, and similar patterns were obtained (results not shown).

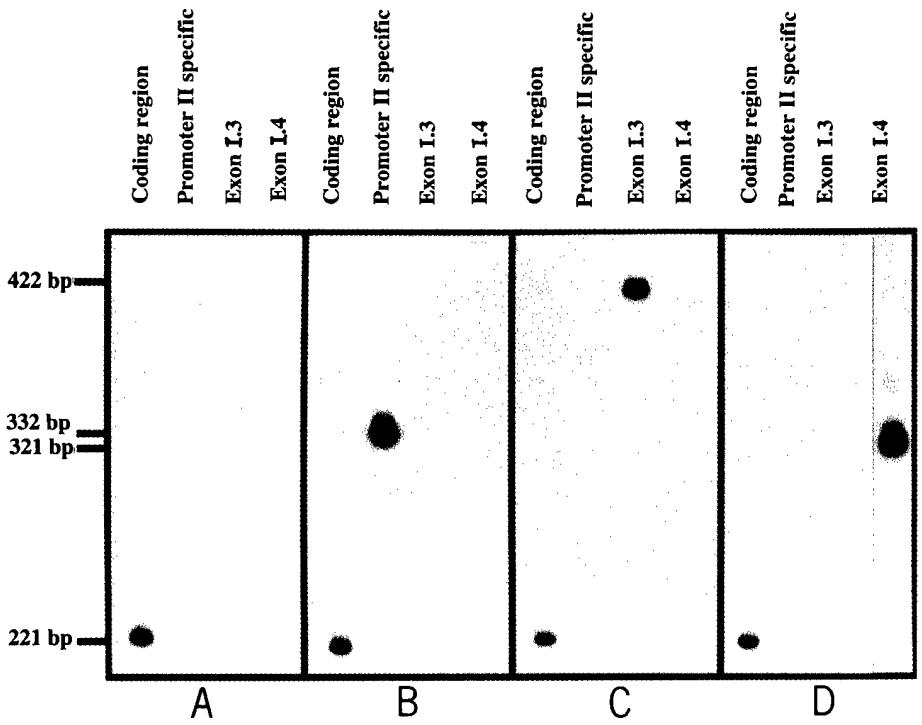


**Fig. 4.** Cross-amplification reactions. Four reactions were set up for each synthetic transcript by using primers specific for coding region, promoter-II-specific sequence, exon I.3 and exon I.4, as indicated. (A) Coding-region-specific transcript; (B) promoter-II-specific transcripts; (C) exon I.3-specific transcripts; (D) exon I.4-specific transcripts.

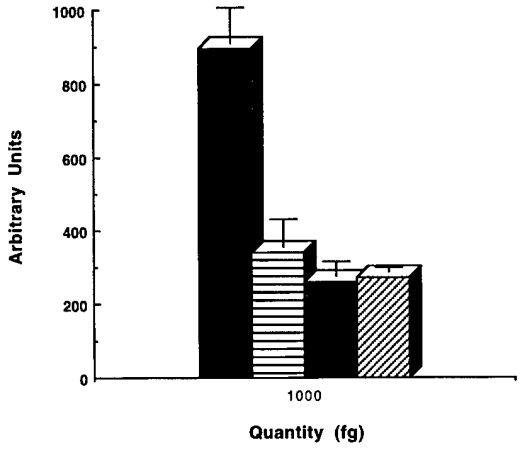


**Fig. 5.** Amplification of each transcript from the mixture of three synthetic transcripts (1 pg of each), employing specific primers. ■, cod; ▤, PII; ▥, I.3; ▨, I.4. Results shown are the mean ± SD of data from three separate experiments.

Results of the experiments using cultured cells are shown in Fig. 8 and Table 5. Quantification of each transcript in the cells was performed using two or three different concentrations of internal standard. The concentration at which amplified products of target and synthetic transcripts were equal was considered the concentration of target transcripts (corrected for size difference because molar ratio is equal). Because of the complexity of the gel, the data showing amplified products from target and internal standard transcripts for each 5′ terminus is not shown but results are presented in Table 5.

In human adipose stromal cells cultured in the absence of serum, the amplification products of promoter II and exon I.3 (truncate)-specific sequences appeared to be similar in abundance. There was no amplification of exon I.4-specific sequences (Fig. 8A). In adipose stromal cells treated with dexamethasone in the presence of serum, we observed good amplification of exon I.4 and I.3 (truncate)-specific sequences. There was very low expression of promoter-II-specific transcripts (Fig. 8B, Table 5). Transcripts containing exon I.4-specific sequences and exon I.3 (truncate)-specific sequences were almost equal in abundance (Table 5). Total P450arom transcript expression (detected by amplifying the coding region) was about four times higher than in adipose stromal cells grown in the absence of serum (Table 5). When human adipose stromal cells were treated with dexamethasone and the cytokine IL-11, total P450arom transcripts were present in a high copy number, that was similar to the abundance of total P450arom transcripts present in cells treated with dexamethasone plus serum (Table 5). All three 5′ termini were amplified (Table 5). Although transcripts containing exon I.4-specific sequences were the most

**Table 4.** Conditions used to standardize quantification of internal standard

| Standard experiments | Amounts of exon-specific synthetic transcripts | | | |
|---|---|---|---|---|
| | Promoter-II-specific | Exon I.3 | Exon I.4 | Sheep kidney |
| 1 | 50–1000 fg | 50 fg | 50 fg | 50 ng |
| 2 | 50–1000 fg | 500 fg | 500 fg | 50 ng |
| 3 | 50–1000 fg | 1000 fg | 1000 fg | 50 ng |
| 4 | 50 fg | 50–1000 fg | 50 fg | 50 ng |
| 5 | 500 fg | 50–1000 fg | 500 fg | 50 ng |
| 6 | 1000 fg | 50–1000 fg | 1000 fg | 50 ng |
| 7 | 50 fg | 50 fg | 50–1000 fg | 50 ng |
| 8 | 500 fg | 500 fg | 50–1000 fg | 50 ng |
| 9 | 1000 fg | 1000 fg | 50–1000 fg | 50 ng |

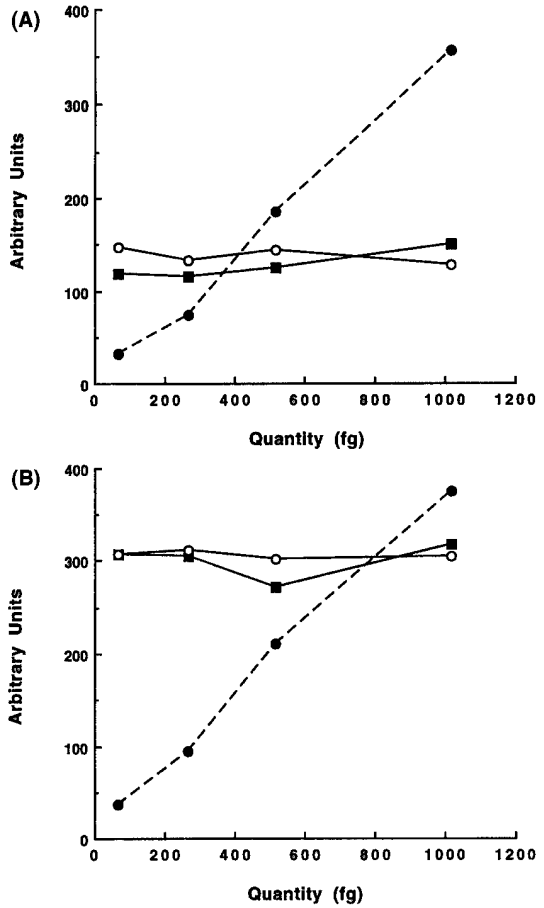


**Fig. 6.** Amplification of mixtures of three transcripts containing varying quantities of promoter-II-specific transcripts in the presence of a fixed amount [(A) 500 fg; (B) 1000 fg] of the other two transcripts, i.e. exon I.3 and exon I.4 (see Table 4 for details of concentration of each transcript in the mix). ●, PII; ■, I.3; ○, I.4.

abundant, promoter-II- and exon I.3 (truncate)-specific transcripts were also present in higher copy number in comparison to cells treated with dexamethasone alone.

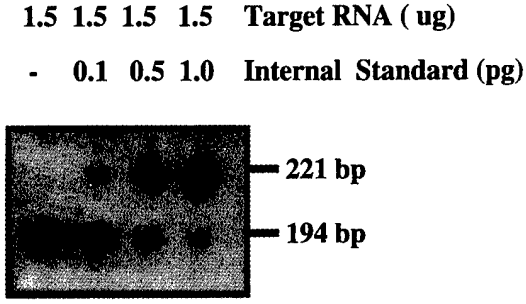


**Fig. 7.** Competitive RT-PCR: 1·5 μg of adipose stromal cell RNA was mixed with 0–1 pg of internal standard transcript (coding-region-specific) and amplified by using coding-region-specific primers. The size of the internal standard amplified product was 221 bp, and that of the target amplified product was 194 bp.

In primary cultures of human ovarian granulosa cells treated with forskolin for 48 h, promoter-II-specific sequences were those mainly amplified (Fig. 8C, Table 5). Transcripts containing exon I.3 (truncate)-specific sequences were in very low copy number (Table 5). Transcripts containing exon I.4 were undetectable.

## DISCUSSION

Tissue-specific expression of the human *CYP19* gene appears to be regulated by tissue-specific promoters in the ovary, placenta and adipose tissue.[22] This conclusion is based on the presence of specific 5′ termini present in transcripts encoding P450arom in each of these tissues. Transcripts specific for proximal promoter-II-specific sequence are found in the ovary, whereas transcripts specific for the distal promoter I.1 are found uniquely in placenta. Adipose tissue contains mainly two species of transcripts with exon I.3 and I.4-specific sequences. On the other hand, when adipose stromal cells are maintained under

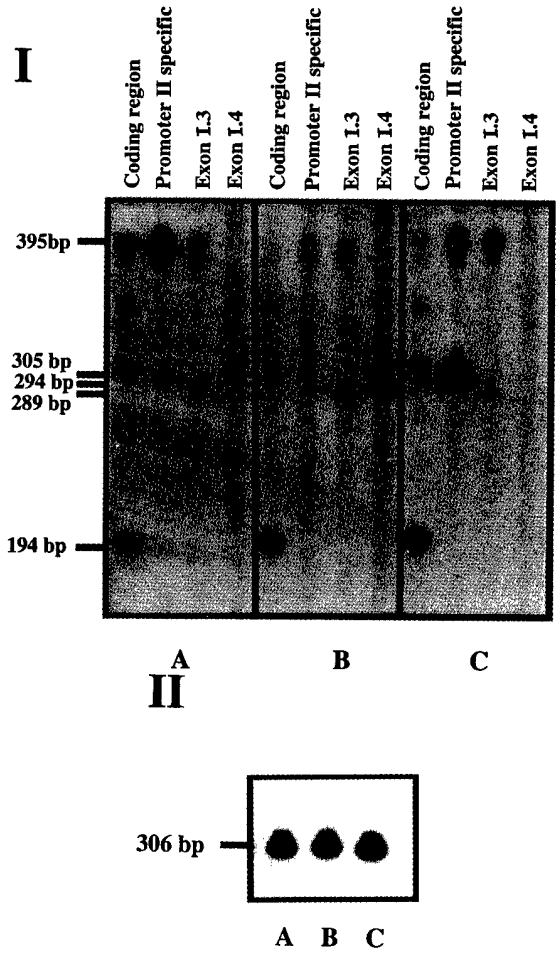


**Fig. 8.** (I) Amplification of specific 5′ terminal sequences from cDNA of 1 μg of RNA isolated from (A) adipose stromal cells maintained in the absence of dexamethasone and serum; (B) adipose stromal cells in the presence of dexamethasone +serum; (C) ovarian granulosa cells in the presence of forskolin. The specific 5′ terminus being amplified is indicated at the top of each lane. (II) Amplification of GAPDH cDNA from 500 ng RNA isolated from cultured cells. Panels A, B, C are as indicated above.

different culture conditions,[23] different 5′ termini are found. Therefore, it became very important to design a very sensitive, rapid and quantitative method to detect multiple alternatively spliced transcripts present in various human tissues and cells grown in different culture conditions. Thus, we designed a quantitative RT-PCR method in which we used four different internal standards (synthetic transcripts) specific for transcripts containing each 5′ terminus, i.e. promoter-II-specific sequence, exon I.3-, exon I.4-containing transcripts, as well as the coding region. Use of internal standards in quantitative RT-PCR has become common these days, but our method of synthesizing internal standards by inserting a 27 bp random sequence in combination with direct cloning using the CloneAmp System (Gibco-BRL) is very rapid, especially since it was necessary to prepare multiple internal standards. Using trace amounts of [α-$^{32}$P] dCTP in the PCR reaction, it was possible to resolve the reactions on a 0·3 mm non-denaturing polyacrylamide gel which was dried and exposed to either X-ray film or phosphorimager for quantification. This technique yielded quick and accurate results, and additionally, it was possible to distinguish amplified bands differing by only 5 or 6 bp.

In addition to standardization of these basic techniques, the aim of our research was to amplify multiple 5′ termini of alternate transcripts using the same reaction conditions, since employing separate reaction conditions for each specific 5′ terminus would have proved difficult for two reasons: (1) Setting up separate PCRs could have resulted in pipetting errors which are more likely to occur for smaller volumes, while this can be minimized by making master mixes. (2) Setting up separate PCRs is time consuming, especially when the sample number is very high. Because we have to amplify the 5′ termini of the transcripts of many samples, to simplify the procedure we decided to normalize one set of PCR conditions for amplification of three 5′ termini, i.e. promoter-II-

**Table 5.** Quantification of multiple alternatively spliced transcripts (copy number $\times 10^6$) present in 1 μg of total RNA from human adipose stromal cells and ovarian granulosa cells maintained in primary culture under different conditions

| Alternative transcripts | Adipose stromal cells | | | Granulosa cells |
|---|---|---|---|---|
| | −Serum −Dexamethasone | Dexamethasone +serum | Dexamethasone* +IL-11 | +Forskolin |
| Promoter-II-specific sequence | 1·2 | 0·12 | 2·4 | 12·0 |
| Exon I.3 | 1·6 | 4·4 | 2·5 | 0·04 |
| Exon I.4 | — | 4·8 | 6·0 | — |
| Coding region | 3·6 | 14·4 | 12·0 | 11·6 |

* This experiment was conducted separately.
These experiments were performed twice yielding similar results. The data presented in the table was obtained from a single experiment.

specific sequences, exon I.3, exon I.4 and the coding region. To check the validity of these reaction conditions, we performed a number of experiments using synthetic transcripts (internal standards). The same primer pairs were able to amplify sequences from both target RNA and synthetic cRNA. The only difference was that products amplified from synthetic RNA were 27 bp larger in size.

Further, when using the same conditions for four sets of primers (specific for each 5′ terminus) in which the antisense oligo was the same, there was a high possibility of cross-reaction. However, no cross-amplification was observed (Fig. 4). Efficiencies of amplifications of the 5′ termini were also checked by mixing equal quantities (1 pg) of synthetic transcripts (Fig. 5). This was very important information, since we were trying to amplify one 5′ terminus in one tube from the mixture of several 5′ termini. We found equal efficiencies of all three 5′ termini amplified. Further, when we amplified coding-region-specific sequences, we observed three times more amplified product as of untranslated exons (Fig. 5). This was the anticipated result, since the coding region amplified product should be the sum of all three transcripts used, as it is common to all synthetic transcripts. The concentration of these transcripts in 1 μg of cultured cells was also in a similar range (Table 5), thus we could conclude that amplification efficiencies of all three untranslated exons were equal in our experiments using human adipose stromal cells and granulosa cells.

Similarly, we performed experiments using various combinations of the three synthetic transcripts containing promoter-II-specific sequence, exon I.3 and exon I.4 (Table 4, Fig. 6). We observed that the efficiency of amplification of one 5′ terminus was not changed in the presence of varying concentrations of the other two 5′ termini. This was a very important point when trying to amplify and quantify multiple 5′ termini from alternatively spliced transcripts present in a tissue. Further, competition experiments were also performed and satisfactory results were obtained (Fig. 7) showing competition between target cDNA and cDNA of internal standard transcripts. Thus, we were able to calculate the amount of unknown target transcript by comparing amplified products with the amplified products of known quantities of internal standard transcripts added in the reaction.

After satisfactory standardization of the PCR conditions, we amplified different 5′ termini of transcripts present in human adipose stromal cells and ovarian granulosa cells in primary culture. Previously we observed that the distribution of the various 5′ termini in adipose stromal cells appeared to be a function of the culture conditions under which the cells were maintained.[23] By Northern analysis, we could detect 5′ termini of only abundantly expressed transcripts. We also amplified 5′ terminal sequences by means of the RACE method,[23] but the observed distribution may not reflect the true quantitative distribution of these termini in the *CYP19* gene transcripts in a particular cell or tissue.

Our present results employing adipose stromal cells grown in the absence of serum or dexamethasone revealed the presence of equal concentrations of transcripts containing exon I.3 (truncate)- and promoter-II-specific sequences. There was no amplification of exon I.4-specific sequences. Results obtained from adipose stromal cells treated with dexamethasone (250 nM) in medium containing 15% FCS are in agreement with our earlier study.[23] Exon I.3 and exon I.4-specific sequences were amplified, but exon I.4-specific sequences were more abundant in dexamethasone-treated cells (Fig. 8). Promoter-II-specific sequences were present in very low copy number. Total levels of P450arom transcripts (see coding region, Fig. 8, Table 5) were elevated relative to control cells, which is in accordance with our earlier observation that, in the presence of serum, glucocorticoids stimulate aromatase activity.[32] Recently, we have shown that the effect of glucocorticoids in the presence of serum is regulated by promoter I.4.[33] More importantly, we found that the stimulatory action of serum (in the presence of dexamethasone) can be replaced by the cytokine IL-11. Moreover, we have shown that this operates via a Jak/STAT signaling pathway to regulate aromatase P450 gene expression by using promoter I.4.[33] Therefore, we decided to amplify different transcripts from adipose stromal cells treated with dexamethasone and IL-11. As expected, we observed a high abundance of exon I.4-specific sequences (Table 5). Interestingly, we also observed amplification of promoter-II-specific and exon I.3 (truncate)-specific sequences (Table 5).

In ovarian granulosa cells treated with forskolin, only promoter-II-specific sequences were amplified (Fig. 8). Exon I.3 (truncate)-specific sequences were present in very low copy number. The presence of promoter-II-specific sequences in cells treated with forskolin is in agreement with our earlier studies based on Northern analysis.[34] Presence of exon I.3 (truncate)-specific sequences could not be detected by Northern analysis because of low copy number.

It seems likely that promoter II and I.3 are involved in basal transcription of the *CYP19* gene in adipose stromal cells. In cells treated with glucocorticoid and IL-11 or serum, total P450arom transcripts are elevated due to increased expression from exon I.4. However, there are also more exon I.3 (truncate)-specific sequences. So possibly there are other factors

which regulate the *CYP19* gene by utilizing promoter I.3. Most interestingly, from these results, we observed that in human adipose stromal cells and ovarian granulosa cells, the exon I.3-specific transcripts being amplified were the truncated version.[23] There was no amplification of full-length exon I.3-specific transcripts. Although the 5′ end primer (RT-2) was chosen from the sequence which was common in both exon I.3 and exon I.3 (truncate) transcripts, the size difference of 106 bp between both amplified PCR products is very clear. Earlier, by RACE cloning, both full-length and truncated exon I.3-specific transcripts were detected in cultured cells.[23] At this point we are unable to explain this difference.

We conclude that by using this very sensitive competitive RT-PCR method, we were able to amplify and quantify P450arom transcripts present in very low copy number. Most importantly, we were able to differentiate amplified products of only 5–6 bp difference in size. This methodology will be very useful to study the regulation of the *CYP19* gene in different tissues and in different disease states. Experiments are already in progress in our laboratory to determine the levels of the various exon-specific transcripts, present in the endometrium of patients with endometriosis and in adipose tissue of breast cancer patients. Furthermore, this procedure is not only useful for detecting alternatively spliced transcripts of the *CYP19* gene, but it can in principal be applied to detect multiple transcripts expressed from other genes which are regulated in this fashion.

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Rapid Paper

# Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells

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**Abstract**

The expression of transcripts of cytokines of the interleukin-6 (IL-6) family has been examined in human breast tumors, breast cancer cell lines, and adipose stromal cells, by means of reverse transcription polymerase chain reaction amplification. Of the six breast tumor samples examined, all expressed transcripts encoding IL-6 and Leukemia Inhibitory Factor (LIF). Four of the samples also expressed transcripts for oncostatin M (OSM) and IL-11, and three expressed the IL-6 receptor. Adipose stromal cells expressed IL-6, IL-11 and LIF, but not the IL-6 receptor, consistent with previous conclusions that IL-6 activity in these cells required addition of IL-6 soluble receptor. In the case of T47D cells, expression of IL-11 protein was confirmed by immunotitration. Moreover, in these cells, expression of IL-11 transcripts was induced 3-fold by addition of estradiol to the culture medium. These results add credence to our previous proposal that breast cancer development is regulated in part by local autocrine and paracrine mechanisms via epithelial/mesenchymal interactions, in which estrogen produced by stromal cells surrounding the tumor acts to stimulate the production of growth factors and cytokines by the tumor cells. Some of these may act to stimulate further the growth and development of the tumor, while these or other factors may act on the surrounding mesenchymal cells in a paracrine fashion to stimulate aromatase expression in the presence of glucocorticoids. Thus, a positive feedback loop is established which leads to the development and growth of the tumor.

*Keywords:* Aromatase; Cytokines; Breast cancer; Adipose

## 1. Introduction

Estrogen biosynthesis is catalyzed by aromatase P450 ($P450_{arom}$, the product of the CYP19 gene). Aromatase activity is found in a variety of tissues including adipose, ovary, placenta, testis and brain [1–6]. In postmenopausal women adipose tissue is the primary site of estrogen biosynthesis [7–10]. Studies from a number of laboratories have examined aromatase activity or else aromatase expression, as determined by RT-PCR, in breast adipose tissue from cancer patients [11–16]. The results have indicated that aromatase expression is higher in adipose tissue proximal to a tumor, as compared to that distal to a tumor. Although such a correlation was not observed in one study [17], these observations suggest that there is crosstalk between the tumor and the surrounding tissue in terms of the ability of the latter to synthesize estrogens, and that factors produced by tumors stimulate the surrounding stromal cells to produce estrogens. These in turn stimulate the tumor cells to produce more growth factors, and thus stimulate the growth of the tumor in a paracrine fashion [18,19].

In order to study the regulation of aromatase expression in adipose tissue, we have utilized a cell model, namely adipose stromal cells in primary culture [20]. Tissue-specific regulation of the $P450_{arom}$ gene is due, in part, to the use of alternative transcriptional start sites as a consequence of tissue-specific promoters [21–25]. In adipose tissue three promoters are used, promoter II, promoter I.3 and promoter I.4 [26,27]. Furthermore, different hormonal conditions will also determine which


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of the three promoters is used in adipose stromal cells in monolayer culture [26]. Glucocorticoids, in the presence of serum, induce the expression of aromatase via promoter I.4. Promoter I.4 is a distal TATA-less promoter which contains an upstream glucocorticoid response element (GRE), an interferon-$\gamma$ activation site (GAS) and an Sp1 site [28]. Reporter gene analysis of this promoter demonstrated that the GRE and Sp1 binding site were required for transcription in the presence of serum and glucocorticoids [28]. Earlier work had demonstrated the requirement for serum in the induction of aromatase activity in the presence of glucocorticoids, however, no good candidate for the 'serum factor' could be found [29]. Recently, conditioned medium from breast cancer cell lines, as well as other cell types, has been found to mimic the action of serum in the stimulation of aromatase by glucocorticoids in adipose stromal cells in culture [30,31], as well as tumor fibroblasts [12]. Similarly, members of the gp130 receptor family of cytokines, namely interleukin-11 (IL-11), leukemia inhibitory factor (LIF), and oncostatin M (OSM), have been shown to replace serum under these circumstances [32]. This suggests that one or more members of this cytokine finally could be present in the conditioned media from these cells. In contrast to the other members of this cytokine family, IL-6 could not induce aromatase activity on its own. However, IL-6 was as active as IL-II and OSM when the IL-6 soluble receptor was added to the culture medium [32]. Previously, Reed and colleagues had shown that IL-6 was present in breast cystic fluid and could stimulate aromatase expression of breast tumor fibroblasts in the presence of dexamethasone [12].

Further analysis of the promoter I.4 region revealed that the GAS element, to which members of the STAT family of transcription factors are known to bind, was essential for cytokine-induced expression of aromatase. Recent findings from this laboratory have shown that stimulation of aromatase by members of this cytokine family and glucocorticoids is mediated by a member of the Jak family of tyrosine kinases (JAK1) as well as a STAT transcription factor (STAT3) which binds to the GAS element [32].

In the work presented here, the expression of these cytokines by adipose stromal cells, a breast cancer cell line and breast tumor samples was evaluated. The results indicate that most of the tumor samples and cell lines tested express one or more of these cytokines. These findings lend further support to the hypothesis that cytokines produced by breast tumors, as well as by adipose stromal cells, may up-regulate the expression of aromatase in surrounding tissue, and the resulting increase in local estrogen production could influence, in turn, the growth of the tumor.

## 2. Material and methods

### 2.1. Culture and treatment of adipose stromal cells

Subcutaneous adipose tissue was obtained from women at the time of reduction abdominoplasty or reduction mammoplasty. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were prepared as described [20] and maintained in primary culture in Waymouth's enriched medium containing 10% Nu serum (10% v/v) (Collaborative Research Inc.) and allowed to grow to confluence (5–6 days) before treatment. At this time serum was removed for 24 h, and the cells were treated with 250 nM dexamethasone for 48 h before cytokines or conditioned medium from T47D cells was added. Aromatase activity was determined by the incorporation of tritium into [$H^3$]water from (1$\beta$-$H^3$]androstenedione as described previously [20].

### 2.2. Culture and treatment of T47D cells

T47D cells were cultured in RPMI (without phenol red), 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were grown to confluence before treatment. At this time serum was removed for 24 h (with two changes of media during this period to ensure complete removal of serum), and the cells were treated with estradiol for 24 h or left untreated. The conditioned medium was collected and centrifuged to remove cellular debris.

### 2.3. Culture of control cell lines

As controls, cell lines were cultured which were reported to express the individual cytokines: U937, a human histiocytic lymphoma cell line, which expresses IL-11 and IL-6; HCT-8/HRT-18, a human ileocecal adenocarcinoma, which expresses LIP; and CESS, a human lymphoblastoma, which expresses IL-6 soluble receptor. All cell lines were obtained from ATCC and cultured according to their instructions.

### 2.4. RNA preparation from cells in culture

RNA was prepared using RNasolB (Biotecx Inc, Houston, TX), according to the manufacturer's instructions.

### 2.5. RNA preparation from tumor samples

RNA was prepared by the guanidinium thiocyanate/cesium chloride method as described [33]. RNA concentration was determined by spectrophotometric absorption at 260 nm.

### 2.6. RT-PCR

RNA was denatured at 65°C for 2 min and annealed to random hexamers (Gibco-BRL) by allowing the samples to cool slowly to 37°C. To synthesize the complementary DNA (cDNA), the annealed RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) at 37°C for 1 h in the presence of 200 $\mu$M of each dNTP, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DDT and 5 units of RNasin (Gibco-BRL). cDNA was amplified using the polymerase chain reaction in the presence of 500 $\mu$M of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin (w/v), 10 pmol of the specific oligonucleotide (see Table 1) and 4 units of *Taq* polymerase (Perkin-Elmer) in a final volume of 20 $\mu$l.

PCR conditions were: denaturing at 94°C for 30 s, annealing at 50°C for IL-6, LIF, OSM and IL-6 receptor and 55°C for IL-11 for 30 s, and extension at 72°C for 1 min, for 25 cycles. The PCR was performed in a Gene Amp PCR system 9600 (Perkin-Elmer). GAPDH sequence was also amplified from each sample as a separate check on amplification efficiency (data not shown). The amplification products were run on a 1.5% agarose gel and transferred to Hybond $N^+$ (Amersham) by capillary elution in 0.4 M NaOH solution. Southern hybridization was carried out with specific oligonucleotides, internal to the sense and antisense oligonucleotides used in the PCR amplification, which were end-labeled with [$^{32}$P]phosphate. Hybridization was carried out overnight at room temperature followed by a series of stringent washes at 5°C below the $T_m$ of the oligonucleotide. Radioactivity on the membrane was determined by autoradiography.

Table 1
Oligonucleotides used for RT-PCR amplification of specific cytokines

| | |
|---|---|
| Interleukin 11 (Sequence described in [37]). | |
| Sense: | 5′-CACATGAACTGTGTTTG CCGCCTGGT-3′ |
| Antisense: | 5′-GCAGCCTTGTCAGCAC ACCTGGGAGCTGTAGA-3′ |
| Internal: | 5′-TCGAGGGGGGCCAGGT GGTGGCCC-3′ |
| Interleukin 6 (Sequence described in [38]). | |
| Sense: | 5′-GCCTTCGGTCCAGTTGC CTTC-3′ |
| Antisense: | 5′-CTGCAGGAACTGGATC AGGAC-3′ |
| Internal: | 5′-AAGGTTCAGGTTGTTTT CTGCCAG-3′ |
| Interleukin 6 soluble receptor (Sequence described in [39]). | |
| Sense: | 5′-ATGCTGGCCGTCGGCT GCGCG-3′ |
| Antisense: | 5′-GAAAGAGCTGTCTCCC TCCGG-3′ |
| Internal: | 5′-CCGGTAGCATGAATAG TTTCCAGA-3′ |
| Oncostatin M (Sequence described in [40]). | |
| Sense: | 5′-ACGCTGCTCAGTCTGG TCCTT-3′ |
| Antisense: | 5′-CACCCCCTTCCTCAGGG CCTG-3′ |
| Internal: | 5′-GATGTTCAGCCCAGAC CTCTCCAA-3′ |
| Leukemia Inhibitory Factor (Sequence described in [41]) | |
| Sense: | 5′-AAACATGGGGCGGGGA GCCCC-3′ |
| Antisense: | 5′-GCACTGGGGTTGAGGA TCTTC-3′ |
| Internal: | 5′-AAAGAGGGCATTGGCA CTGCCATT-3′ |

## 3. Results

### 3.1. Reverse transcriptase polymerase chain reaction analysis of breast tumor samples, breast cancer cell lines and adipose stromal cells

The RT-PCR analysis is shown in Fig. 1 and a summary of the results is given in Table 2. The RT-PCR method enables a range of tumor samples and cell lines to be assessed for the expression of transcripts for a particular cytokine. All the tumor samples expressed one or more of the cytokines examined, namely IL-6, IL-11, LIF, OSM, as well as the IL-6 receptor, with wide variation in the pattern and levels observed between patients. Adipose stromal cells expressed all the transcripts except IL-6 receptor and OSM. T47D cells expressed only IL-11 and IL-6 receptor. Estradiol ($10^-$ 7 M) induced the expression by T47D cells of IL-11 transcripts, but not of IL-6 receptor.

### 3.2. The effect of conditioned media from estradiol-treated T47D cells on aromatase activity in adipose stromal cells in culture

As can be seen from Fig. 2, conditioned media from T47D cells stimulated $P450_{arom}$ activity of adipose stromal cells maintained in the presence of dexamethasone. The stimulatory factor(s) present in the conditioned media was completely titrated by addition of an anti-IL-11 antibody in a concentration dependent fashion. However, the antibody had no effect on basal activity in the absence of conditioned medium.

## 4. Discussion

From the results of the present study, it is apparent that of the six breast tumor samples examined, all were capable of expressing transcripts for LIF and IL-6. Of the six, four were also capable of expressing transcripts for oncostatin M and IL-11. Moreover, three of the

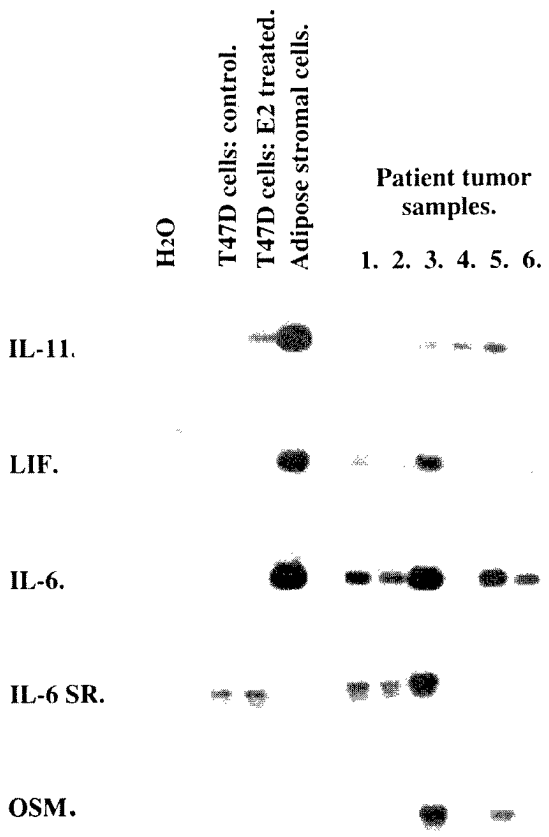


Fig. 1. Reverse transcriptase polymerase chain reaction analysis of breast tumor samples, T47D cells and adipose stromal cells. RNA was prepared using the guanidinium thiocyanate-cesium chloride method. The RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase. Specific oligonucleotides for the polymerase chain reaction were designed to amplify portions of the respective cDNAs, which spanned exons within each gene. After PCR the samples were run on a 1.5% agarose gel, then transferred to nitrocellulose. In order to identify specific PCR products, the nitrocellulose was probed with oligonucleotides complementary to specific sequence for the gene and internal to those used for amplification.

tumor samples expressed the receptor for IL-6. Thus it would appear that breast tumors are capable of synthesizing factors which stimulate aromatase and enhance estrogen production by surrounding adipose stromal cells, although obviously caution must be exercised in extrapolating from an expressed transcript to a secreted protein. Interestingly, a breast cancer cell line, namely T47D, was capable of expressing only transcripts for IL-11 and the IL-6 receptor. However, the expression of IL-11 transcripts was induced by treatment of the cells with estradiol, although expression of the IL-6 receptor was not. Previously we observed that conditioned medium from T47D cells was capable of stimulating aromatase activity by adipose stromal cells [30]. This stimulatory activity was increased by estradiol in a concentration-dependent fashion [30], and moreover could be blocked by an antibody to IL-11 (Fig. 2). Thus it is likely that the factor present in T47D cell-conditioned media which stimulates aromatase activity of adipose stromal cells is IL-11, and moreover its expression and secretion by the T47D cells is stimulated by estrogens. So in this case, at least, expression of the transcript results in secretion of a bioactive protein.

If extrapolated to the in vivo situation, these results add credence to our previous proposal that breast cancer development is regulated in part by local autocrine and paracrine mechanisms via epithelial/stromal interactions, in which estrogen produced by stromal cells surrounding the tumor acts to stimulate the production of growth factors and cytokines by the tumor cells. Some of these may act to stimulate further the growth and development of the tumor, while these or other factors may act on the local adipose stromal cells in a paracrine fashion to stimulate aromatase expression in the presence of glucocorticoids. Thus, a positive feedback loop is established which leads to the development and growth of the tumor.

An important unanswered question relates to the nature of the cell type(s) within the tumor samples which are expressing these cytokines. In a recent study, Purohit et al [19] stress the importance of infiltrating macrophages in the production of cytokines such as IL-6 at breast tumor sites. In a study of a patient with a chronic inflammatory response to silicon injection for the purposes of breast augmentation, aromatase activity in breast tissue was very high and correlated with IL-6 production [19].

From the results of the present study, it is apparent that adipose stromal cells themselves have the capacity to express a number of these cytokines, although they do not express the receptor for IL-6, consistent with previous observations that IL-6 has no effect to stimulate aromatase expression by these cells, except when the soluble receptor is added [32]. However, the fact that the cells can also express IL-11 and LIF, raises the possibility that aromatase expression by adipose stromal cells themselves is regulated in part by paracrine and autocrine mechanisms involving cytokine secretion in the absence of any breast tumor or other heterologous cell type. It is conceivable therefore that a major factor regulating aromatase expression in adipose is local production by adipose cells of such stimulatory factors. Since IL-6 levels in plasma are reported to be increased with aging in both humans [34] and rodents [35], it is conceivable that the previous observation of increased adipose tissue aromatase expression with aging [36] may also be the consequence of production of aromatase-stimulating cytokines, either in the adipose tissue itself or in other body sites.

Table 2
Summary of expression of cytokines in breast tumor samples, T47D breast cancer cells and adipose stromal cells, as determined by RT-PCR

| Cell line | | Expressed product | | | | |
|---|---|---|---|---|---|---|
| | | IL-11 | LIF | IL-6 | IL-6R | OSM |
| T47D | Control | + | − | − | + | − |
| | E2 treated | ++ | − | − | + | − |
| Adipose stromal cells | | ++++ | +++ | ++++ | − | − |
| Patient tumor samples | | | | | | |
| | 1. | − | + | ++ | ++ | − |
| | 2. | − | + | ++ | + | − |
| | 3. | + | ++ | +++ | +++ | +++ |
| | 4. | + | + | + | − | + |
| | 5. | ++ | + | ++ | − | ++ |
| | 6. | + | + | ++ | − | + |

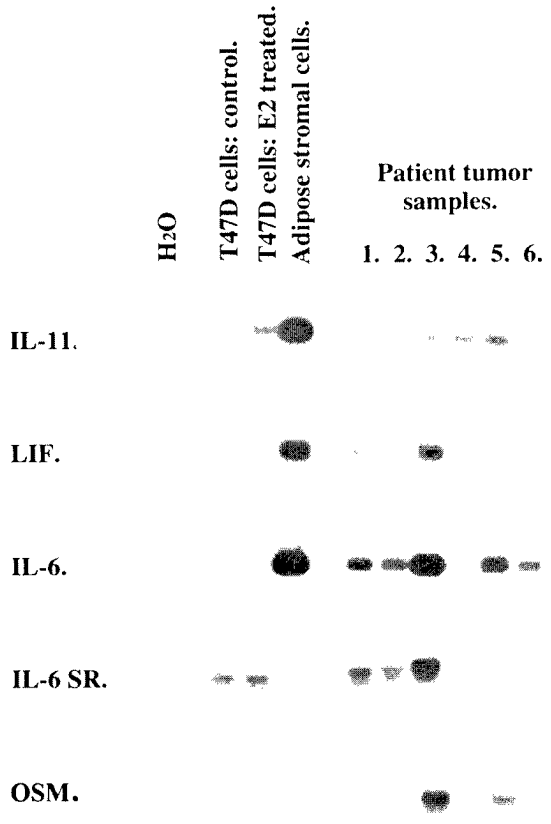


Fig. 2. The effect of conditioned media from estradiol-treated T47D cells on aromatase activity in adipose stromal cells in culture. T47D cells were grown to confluence, then maintained for two successive 12-h periods in serum-free medium, after which time serum-free medium was left on the cells for 24 h, then removed and applied to the adipose stromal cells in primary culture, in the presence or absence of anti-IL-11 antibody in various concentrations. Aromatase activity was then determined following a 2-h incubation with [1$\beta$-$^3$H]androstenedione. CM: conditioned medium from T47D cells applied to adipose stromal cells. DEX: dexamethasone. Results are expressed as pmol [$^3$H]water formed per mg protein per 2 h, and represent the mean ± S.E.M. of triplicate replicate dishes.

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# Cellular Characterization of Adipose Tissue from Various Body Sites of Women*

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**ABSTRACT**

Adipose tissue is the primary site of estrogen biosynthesis in postmenopausal women. The two main histologic components of adipose tissue are mature adipocytes and fibroblasts. Aromatase P450 expressed in the fibroblast component of adipose tissue is responsible for catalyzing conversion of $C_{19}$ steroids to estrogens. We previously have demonstrated that, in women, aromatase expression in adipose tissue of various body sites increases with age and that aromatase expression in the hip is markedly higher than in the abdomen. To determine whether this age- and regional-dependent variation in aromatase expression is caused by an alteration in the ratio of fibroblasts to mature adipocytes, we collected sc adipose tissue samples from 19 women (age range: 21–93 yr) at the time of autopsy. Using a computerized image analysis system, we determined by morphometry the proportions of adipocytes, fibroblasts, and vascular endothelial cells within histologic sections of adipose tissue from midabdomen, both breasts, and both hips. The percentage of each cell component at each body site was expressed as the mean of triplicate replicates. Statistical analysis of our results did not indicate any correlation between advancing age and fibroblast to adipocyte ratios in the breast, abdomen, or hip. Fibroblast to adipocyte ratios were found to be significantly higher in the breast and abdomen compared with the hip ($P < 0.05$). No statistical differences were found between the breast and abdomen. These findings suggest that the increase in aromatase expression with advancing age and the higher aromatase expression in the hip compared with the abdomen in women may be caused by alterations in specific signal transduction mechanisms rather than a simple increase in local adipose fibroblast numbers. (*J Clin Endocrinol Metab* **81:** 2443–2447, 1996)

ESTROGENS have diverse actions at different body sites of a woman. Estradiol is produced in the ovarian granulosa cells of premenopausal women, whereas estriol and estradiol are secreted by the placenta (1). In both women and men, estrone is produced in the adipose tissue (2), and a substantial part of this estrone is further converted into estradiol in the periphery (3, 4). Aromatase P450 ($P450_{arom}$) is responsible for conversion of $C_{19}$ steroids to estrogens in all of these tissues (5). Adipose tissue is the major source of estrogen synthesis in men and postmenopausal women (3, 6, 7). This occurs primarily by aromatization of circulating androstenedione from the adrenal cortex. In both women and men, MacDonald and co-workers (8) have observed a progressive increase in the efficiency of the extraglandular conversion of circulating androstenedione to estrone with advancing age and obesity. Subsequently, a direct correlation between increasing age and levels of specific adipose aromatase activity in abdominal adipose fibroblast cultures of women has been demonstrated (9). Finally, we have shown that $P450_{arom}$ transcript levels in adipose tissue biopsies from the abdomens, hips, and thighs of women increase with advancing age regardless of body fat mass (10). We also have demonstrated clear regional differences in $P450_{arom}$ transcript levels, in that the levels were the highest in the hip, followed by the thigh, and lowest in the abdomen (10).

Adipose tissue is composed of mature adipocytes, fibroblasts, and vascular endothelial cells. In addition, glandular tissue dispersed within the adipose tissue is found in the breast. Estrogen production in adipose tissue occurs primarily in the nonadipocyte fraction of the adipose tissue (2, 11). In this nonadipocyte fraction, fibroblasts (12, 13) and vascular endothelial cells (our unpublished observations) were immunohistochemically demonstrated to express aromatase. Fibroblasts comprise 87% of the nonadipocyte fraction (11), whereas only 7% of this fraction is accounted for by vascular endothelial cells (11). Fibroblasts are presumed to be preadipocytes that are the precursors for mature adipocytes. We have previously shown that aromatase activity in adipose fibroblasts in culture is regulated primarily by changes in the level of messenger RNA-encoding $P450_{arom}$ (14). Two recent studies from our laboratory have provided evidence that adipose tissue aromatase expression in the breast bearing a tumor (15) and in the disease-free breast (16) is, in part, determined by local fibroblast populations. Regional fibroblast to adipocyte ratios in the breast were found to be directly proportional to adipose tissue $P450_{arom}$ transcript levels. These findings collectively prompted the current investigation, in which we investigated whether the increase in estrogen synthesis in the periphery, as a function of age

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and body site, is also related to increased numbers of fibroblasts in the adipose tissue. To achieve this, we used computerized morphometry to determine the distribution of cellular components of the adipose tissue samples as a function of age and body site.

## Materials and Methods

### *Acquisition of human tissues*

Adipose tissue samples were collected at the time of autopsy from 19 women between the ages of 21 and 93 yr from a variety of ethnic backgrounds. This study has been approved by the Human Institutional Review Board of the University of Texas Southwestern Medical Center. Subjects are characterized in Table 1 as to age, height, weight, and BMI (body mass index = weight/height$^2$). BMI was included as an indirect measure for body fat content. Three adipose tissue samples, each measuring 1 × 1 × 1 cm, were taken at 2 cm from each other in each of five body sites: right and left breasts (4 cm from the skin surface around the projection of the nipple), right and left hips (4 cm from the skin surface in the middle of each hip), and abdomen (4 cm from the skin surface in the midline). Therefore, at least 15 adipose tissue samples were obtained from each subject. Samples in triplicate were also taken from the lower tip of the omentum in 8 women.

### *Histologic morphometry*

Specimens were immediately fixed in 10% neutral buffered formalin. Samples remained in formalin for 24 h before making hematoxylin- and eosin-stained slides. Each slide was examined at 150× magnification. A computerized image analyzer (NIH Image V1.54) was used to achieve random sampling as described by Gunderson and Jensen (17, 18). Fields to be examined were chosen randomly. A grid was overlaid on each field examined and cells landing on intersections of the grid were counted. Mature adipocytes were defined as differentiated cells distended with lipid material. They contain peripherally displaced inconspicuous nuclei (Fig. 1). Fibroblasts, on the other hand, are spindly cells with eosinophilic cytoplasms and dark and elongated nuclei (19). Fibroblasts in adipose tissue are either clustered in connective tissue or dispersed between adipocytes (Fig. 1). A cell was counted as a fibroblast only if the eosinophilic cytoplasm was clearly identified. Endothelial cells are flattened fibroblast-like cells (Fig. 1C) that line the capillaries or small lymphatic vessels (20). In breast adipose tissue samples, epithelial cells lining occasional glandular structures were noted and counted as glandular cells. The quantity of each of the above cell types was expressed as a percentage of the total area. The three samples from each body site were averaged. The results were expressed as the mean ± SEM. The SEM of triplicate samples ranged from ±0.1 to ±3.9% with 54% of SEM less than ±1.0%. The ratio of fibroblasts to adipocytes was calculated and plotted against age, weight, and BMI. Percentage of fibroblasts and percentage of fibroblasts plus endothelial cells were also plotted against age, weight, and BMI.

### *Statistical analysis*

Simple linear correlation analysis and two-way parametric ANOVA followed by Newman-Keuls multiple comparisons procedure were used as statistical methods.

## Results

Table 2 describes the means ± SEM and the ranges of each cell type in 19 subjects. Each cell type is expressed as a percentage of total cells counted for three body sites. The percentages of fibroblasts in the abdominal, left, and right breast samples are greater than those found in the left or right hip samples. As shown in Table 3, no statistically significant correlations were found between the cellular composition of the adipose tissue (in the breast, hip, or abdomen) and age or body fat content of women (n = 19). For the significance level of $\alpha$:0.05, correlation coefficients for none of the body sites exceeded the minimum absolute R value, which is 0.456 for a sample size of 19 (21). These results did not change when patients at the extreme ends of the BMI spectrum (2nd, 4th, 7th, 11th 16th, 17th, and 19th patients, Table 1) were excluded. The fibroblast to adipocyte ratio in the omentum seemed to decrease in women (n = 8) with advancing age; however, this negative trend did not reach the level of statistical significance (minimum significant R value = 0.707).

Two-way parametric ANOVA indicated a statistically significant difference between the percentages of fibroblasts within the adipose tissue or fibroblast to adipocyte ratios in the breast, abdomen, and hip [p(F) = 0.029]. Fibroblast to adipocyte ratios in both the abdomen and breast are significantly higher than those found in the hip (Fig. 2). Newman-Keuls multiple comparisons analysis showed statistically significant differences between the breast and hip ($P < 0.05$), as well as between the abdomen and hip ($P < 0.05$) but not

**TABLE 1.** Characterization of women by age, height, weight, BMI, and race

| Age | Height (in.) | Weight (lbs.) | BMI | Race | Cause of Death | Medical History |
|---|---|---|---|---|---|---|
| 21 | 63.0 | 187 | 33 | L | H | alcohol/cocaine abuse |
| 23 | 64.0 | 330 | 55 | A | U | seizure disorder |
| 26 | 65.0 | 149 | 25 | C | U | UR |
| 27 | 64.0 | 110 | 19 | C | H | UR |
| 32 | 65.5 | 152 | 25 | C | H | UR |
| 36 | 67.0 | 202 | 31 | A | CA | UR |
| 38 | 60.0 | 220 | 42.5 | A | MI | CHD |
| 38 | 63.5 | 130 | 22.5 | A | CA | UR |
| 45 | 68.0 | 140 | 21 | A | OD | cocaine/heroin abuse |
| 45 | 65.5 | 187 | 30 | C | OD | alcohol/cocaine abuse |
| 50 | 63.5 | 207 | 36 | C | L | liver cirrhosis |
| 54 | 65.0 | 168 | 27.5 | C | MI | CHD |
| 58 | 61.0 | 130 | 24.5 | A | CA | UR |
| 61 | 66.0 | 212 | 34 | C | MI | CHD |
| 71 | 59.5 | 112 | 22.5 | L | MI | insulin dependent diabetes |
| 74 | 61.0 | 210 | 39 | C | CA | peripheral vascular disease |
| 74 | 59.5 | 182 | 37 | C | CA | hypertension |
| 76 | 63.5 | 142 | 24.5 | C | MI | CHD |
| 93 | 62.0 | 106 | 19 | C | CA | UR |

BMI, body mass index; weight/height$^2$. Race: A, African-American; C, Caucasian; L, Latin-American. H, homicide; U, unknown; CA, car accident; MI, myocardial infarction; OD, cocaine overdose; L, liver failure; UR, unremarkable; CHD, coronary heart disease.

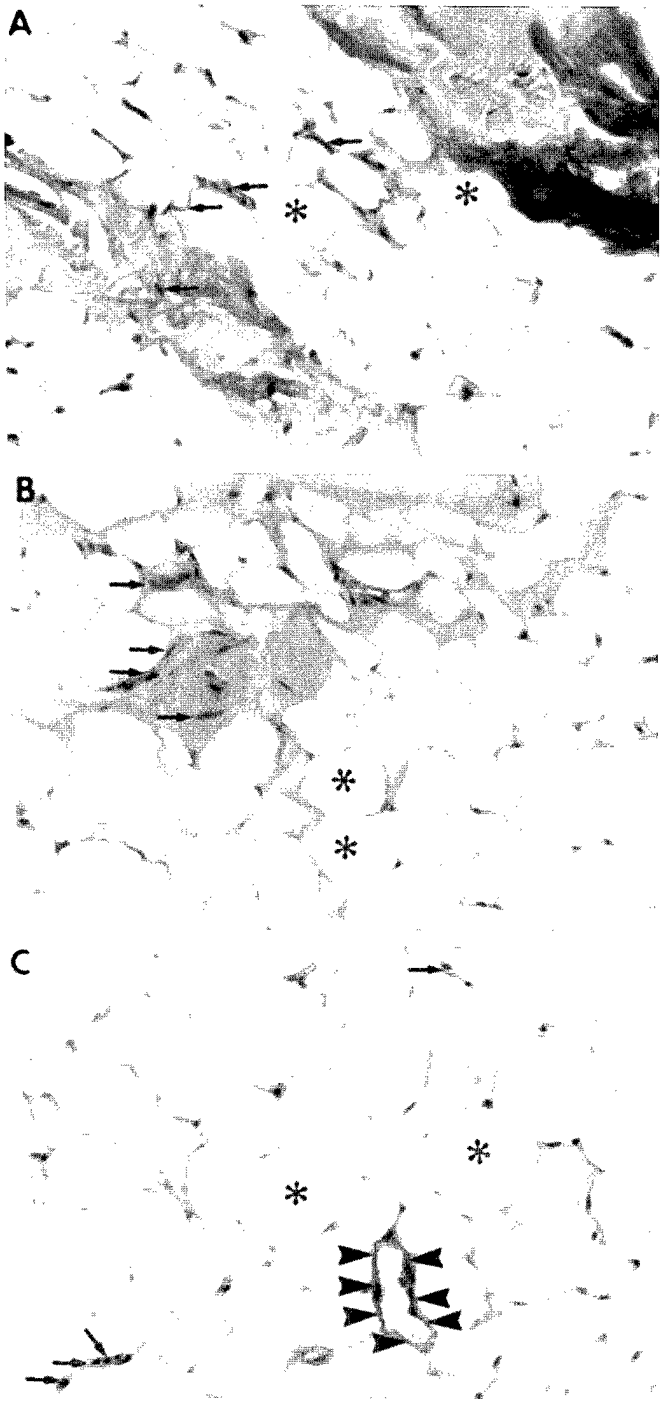


FIG. 1. Hematoxylin and eosin-stained slides of adipose tissue samples from A) breast; B) abdomen; and C) hip. Breast and abdominal samples show relative abundance of fibroblasts (*black arrows*) seen in clusters within dense connective tissue or dispersed among mature adipocytes (*asterisks*). On the other hand, hip sample (C) contains less fibroblasts. Rare endothelium-lined vascular spaces were noted in adipose tissue samples. *Arrowheads* outline a vessel lined with endothelial cells (C).

between the breast and abdomen. This is represented graphically in Fig. 2.

Figure 1, A and B, is hematoxylin and eosin (H&E)-stained slides from breast and abdominal samples, respectively. Figure 1C is an H&E-stained slide from a hip sample. This figure demonstrates the greater fibroblast to adipocyte ratio seen in breast and abdomen samples compared with hip sample.

**TABLE 2.** Mean ± SEM and range (expressed as a percentage of total cells) for each cell component found in body regions of 19 women

| | | Abdomen | Hips | Breasts |
|---|---|---|---|---|
| FB | range: | 1.7–10% | 1.7–9.3% | 1.7–11.7% |
| | mean ± SEM: | 5.91 ± 0.41 | 4.92 ± 0.41 | 6.07 ± 0.52 |
| Ad | range: | 88.9–98% | 89–98.3% | 88.7–98.6% |
| | mean ± SEM: | 92.78 ± 0.51 | 94.02 ± 0.5 | 92.54 ± 0.6 |
| FB/Ad | range: | 0.017–0.112 | 0.017–0.104 | 0.024–0.136 |
| | mean ± SEM: | $6.42 \times 10^{-2} \pm .47 \times 10^{-2}$ | $5.27 \times 10^{-2} \pm .47 \times 10^{-2}$ | $6.63 \times 10^{-2} \pm .61 \times 10^{-2}$ |
| EC | range: | 0.3–3.3% | 0.0–4.3% | 0.0–4.3% |
| | mean ± SEM: | 1.32 ± 0.2 | 1.06 ± 0.2 | 1.30 ± 0.2 |

FB, fibroblasts; Ad, adipocytes; FB/Ad, ratio of fibroblasts to adipocytes; EC, vascular endothelial cells.

**TABLE 3.** Correlation coefficient for age, weight, and BMI plotted against percentage of FB, percentage of FB + EC, and ratio of FB to adipocytes from six different body sites

| | R values | | | | | |
|---|---|---|---|---|---|---|
| | A | LH | RH | LB | RB | O |
| FB *vs.* age | +.184 | −.272 | +.141 | +.110 | −.321 | −.636 |
| FB + EC *vs.* age | +.182 | −.303 | +.055 | +.045 | −.033 | −.643 |
| FB/Ad *vs.* age | +.200 | −.265 | +.141 | +.063 | −.348 | −.589 |
| FB *vs.* wt. | −.263 | −.077 | 0 | −.358 | −.255 | +.411 |
| FB + EC *vs.* wt. | −.283 | −.095 | 0 | −.261 | −.288 | +.456 |
| FB/Ad *vs.* wt. | −.333 | −.110 | 0 | −.356 | −.228 | +.392 |
| FB *vs.* BMI | −.330 | −.118 | 0 | −.366 | −.288 | +.279 |
| FB + EC *vs.* BMI | −.359 | −.110 | +.032 | −.311 | −.336 | +.361 |
| FB/Ad *vs.* BMI | −.390 | −.158 | 0 | −.374 | −.265 | +.243 |

R, correlation coefficient. A, LH, RH, LB, RB, O refer to body site from which specimens were taken and cell numbers counted. A, abdomen; LH, left hip; RH, right hip; LB, left breast; RB, right breast; O, omentum; FB, Fibroblasts; EC, vascular endothelial cells; FB/Ad, ratio of fibroblasts to adipocytes; wt., weight BMI, (body mass index) weight/height$^2$. All cell counts were expressed as percentages.

## Discussion

Specific aromatase expression per unit adipose tissue increases with advancing age irrespective of total body fat mass (9, 10). It has also been demonstrated that aromatase expression resides in the fibroblast component of adipose tissue (2, 11). The next natural question is to ask whether this age-dependent increase in aromatase expression is caused by a change in fibroblast to adipocyte ratio in women. In the present study, we did not find any significant correlation between advancing age and fibroblast to adipocyte proportions in breast, abdomen, and hips, suggesting that alterations in adipose fibroblast numbers is not a mechanism responsible for increased estrogen production with advancing age. The population we studied encompassed a wide age range (21–93 yr) and a sample size (n = 19) large enough, at least, to indicate a trend in reference to our previous study, which included 10 women (10), demonstrating a statistically significant relationship between age and $P450_{arom}$ transcript levels. It should be pointed out here that certain steroid hormones, *e.g.* estradiol and glucocorticoids, can affect body

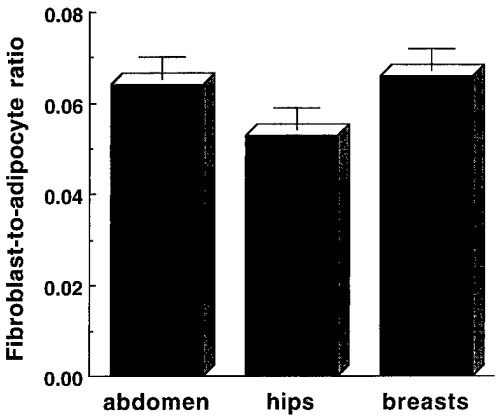


FIG. 2. Graph representing fibroblast to adipocyte ratios in three body sites.

fat distribution. However, the *in vivo* effects of steroids on the cellular composition of body fat are not known. Nonetheless, none of the subjects in the present study was known to have glucocorticoid excess. Exclusion of subjects with extreme obesity to rule out estrogen excess also did not change our results.

Another unexpected result was the fibroblast content of body sites. We had anticipated that fibroblast numbers might follow $P450_{arom}$ transcript levels (10) in that the highest fibroblast to adipocyte ratios would be found in the hip. However, the present study indicated that both breast and abdominal tissue samples showed greater fibroblast to adipocyte ratios than did hip samples. This suggests that variation in adipose tissue aromatase expression between body sites is not mediated by changes in local fibroblast to adipocyte ratios. Mechanisms other than fibroblast content of adipose tissue must be responsible for region- and age-dependent changes in aromatase expression. Areas with the highest fibroblast to adipocyte ratios in tumor-bearing breasts (15) and in disease-free breasts (16) have been shown to contain the highest $P450_{arom}$ transcript levels. Thus, variations in aromatase expression within a region (such as breast, abdomen, and hips) may reflect changes in the ratios of these cell types within that region, whereas variations from one region to another, *e.g.* differences between hips and abdomen, may be related to changes in the specific expression of aromatase in fibroblasts from these sites.

Two mechanisms that may contribute to age-dependent increases in specific aromatase expression, as well as regional variation in expression, may be: 1) increased responsiveness of fibroblasts to hormones or growth substances that stimulate $P450_{arom}$ expression or increased concentration of such factors; and/or 2) decreased responsiveness to inhibitory mechanisms. Thus far, members of the interleukin (IL)-6 cytokine family were found to be the most potent stimulators of aromatase expression in adipose fibroblasts in culture (22). This induction requires the presence of glucocorticoids in the medium (22). Local cortisol levels in the adipose tissue regulated by 11$\beta$-hydroxysteroid dehydrogenase recently have been shown to regulate aromatase activity of adipose fibroblasts (23). Changes in local production of cytokines and cortisol levels are candidate mechanisms for age- and region-dependent increases in estrogen production in adipose tissue. For example, circulating IL-6 levels are elevated in elderly mice and humans, compared with young controls (24, 25). Cyclic adenosine monophosphate analogues also stimulate aromatase expression in adipose fibroblasts; however, this induction seems to be inhibited by serum or growth factors (14). Therefore, any combination of the above mechanisms may be responsible for age- or body site-dependent variation in aromatase expression.

*In vitro* studies have shown that a number of substances influence differentiation of preadipocytes (fibroblasts) to mature adipocytes. Stimulatory substances include the adipogenic transcription factors peroxisome proliferator-activated receptor-$\gamma$ (PPAR$\gamma$) and CCAAT/enhancer binding protein-$\alpha$ (C/EBP$\alpha$) (26). On the other hand, IL-11 (27), tumor necrosis factor-$\alpha$, and C/EBP-homologous protein (28) are capable of inhibiting differentiation of a murine preadipocyte cell line. Also of interest is that IL-11 (22) and tumor necrosis factor-$\alpha$ (our unpublished observations) stimulate aromatase expression in human adipose fibroblasts. Effects of estrogen on expression of these substances are not known yet. Finally, their physiologic roles in determination of cellular composition of human adipose tissue are presently not known.

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